DIFFERENCES IN METABOLISM OF REICHSTEIN'S SUBSTANCE S IN NORMAL ADULTS AND CHILDREN [1]

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The excretion of "corticoids" in the urine of human subjects remains relatively constant throughout life, when referred to body surface area. The excretion of 17-ketosteroids in the urine, however, progressivly rises during adolescence (1).

In the adolescent male, increased 17-ketosteroid excretion is in part attributable to developing testicular function. It is believed that in both sexes increased adrenocortical secretion at puberty is also responsible, although the exact nature of the alteration which occurs is unknown. Talbot, Zygmuntowicz, Wood, and Christo have suggested that a "second" adrenocorticotropic hormone, specifically responsible for the stimulation of 17-ketosteroid production, appears during this era of human life (1).

The increased pubertal excretion of 17-ketosteroids derived from the adrenal cortex may also reflect altered extra-adrenal metabolism of steroids. Certain of the C-21 adrenocortical steroids are known to be degraded to 17-ketosteroids in the body. The greater quantities of urinary 17-ketosteroids appearing with the attainment of maturity might then arise from increased conversion of C-21 steroids to such metabolites. In order to test this hypothesis, Δ^4-pregnene-17α,21-diol,3,20-dione (hereafter designated as Substance S) was administered by various routes to children and adults, and the excretion of 17-ketosteroids and other metabolites was measured. One of the major urinary metabolites of this compound is the 17-ketosteroid, etiocholanolone (2).

METHODS

The subjects were healthy adult volunteers and children from the wards of the Children's Hospital of Philadelphia. The children were convalescent from brief, acute minor illnesses and were well at the time of the study. A 24 hour collection of urine was obtained before and after the administration of Substance S. The urine was collected under a small volume of toluene and stored at 6° C. All urines were examined within one week of collection.

For the measurement of 17-ketosteroids, two 10 ml. aliquots of urine were hydrolyzed, one with 12 per cent (v/v) sulfuric acid (added as 40 per cent sulfuric acid) at 80° C. for 10 minutes, and the other with beta glucuronidase [2] (beef liver), 250 units per ml. for 48 hours at 37° C. Each was extracted with ether and washed (3). The Zimmermann reaction was performed on a small fraction, equivalent to 1.0 ml. of urine, according to the technique previously described (4), modified after Wilson (5). Total urinary corticoids were measured by the method of Silber and Porter (6), with the use of suitably smaller aliquots when the levels were extremely high. Pregnanetriol (pregnane-3α,17α,21-triol) was estimated by the method of Bongiovanni and Eberlein (7).

The formaldehydogenic steroids were determined as follows: Ten ml. aliquots of urine were hydrolyzed with beta-glucuronidase (500 units per ml., at pH 4.5, 37° C., for 48 hours) and extracted three times with equal volumes of ethyl acetate. The combined extract was washed twice with one-twelfth volume of 0.1 N NaOH, and twice with one-twelfth volume of water. All washes were re-extracted with ethyl acetate. After evaporation of the ethyl acetate, the residue was treated with Girard's reagent T in the manner of Schneider (8) and only the nonketonic portion was investigated. The nonketonic residue was partially purified by application to a silica gel column (0.8 × 3.0 cm.) in 2 per cent ethanol/methylene chloride. After washing the column with 10 ml. each of 2 per cent ethanol and 3 per cent ethanol/methylene chloride, the eluate after the application of 25 ml. 10 per cent ethanol/methylene chloride was collected and evaporated at 40° C. with an air current. Suitable aliquots of this fraction were examined by periodate oxidation for nonketonic formaldehydogenic steroids (9).

In one experiment, detailed below, the pooled urine from two subjects was hydrolyzed with beta-glucuronidase, ex-

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[2] Ketodase®, Warner-Chilcott.

tracted three times with one-third volume of ethyl acetate by hand, and washed in the manner described for the smaller aliquots. The extract was chromatographed on silica gel (10). Another pool of urine, specified below, was similarly hydrolyzed, extracted and resolved by chromatography on alumina (11). Both chromatograms were carried to completion within 24 hours.

Substance S was administered in two forms: as the hemisuccinate, by mouth and intravenously; and as the acetate, by mouth only. The single dose of hemisuccinate administered orally was 4.5 to 5.5 mg. per Kg. (expressed as weight of the free steroid), given at the beginning of the second 24 hour collection of urine. In all other instances, the amount of the steroid, as the hemisuccinate by vein or the acetate by mouth, was 1.0 to 1.5 mg. per Kg., administered as a single dose. The hemisuccinate and acetate were prepared by standard procedures (12). In three adult subjects, the acetate of Δ^4-pregnene-11β,17α,21-triol-3,20-dione (hereafter designated "hydrocortisone") was administered orally for purposes of comparison.

RESULTS

The urinary excretion of 17-ketosteroids (after glucuronidase hydrolysis alone), corticoids, and formaldehydogenic steroids, before and after the administration of Substance S, is shown in Table I. Certain subjects were used repetitively. The formaldehydogenic steroids were determined only after the use of the acetate of Substance S. The quantities of pregnane-3α,17α,21-triol excreted were within normal limits for all subjects (7) and did not change. In Table II, the results are expressed as the percentage of the dosage excreted in the form of the particular group of urinary steroids measured; these were calculated from the differences of the urinary levels on the two days of the experiments, and are based on the total dosage of *free* steroid administered. The K/C ratio rep-

TABLE I

Effect of Substance S on urinary steroid excretion

Subject	Age	Sex	Compound	Dose	17-Ketosteroids		Corticoids		Formaldehydogenic	
					C*	A†	C	A	C	A
	yrs.			*mg. free*	*mg./day*		*mg./day*		*mg./day*	
G. W.	5 mos.	M		19.4	0.5	0.5	0.43	2.55		
S. M.	3	M	S‡	58.0	0.15	0.85	1.10	7.20		
C. O.	6	F	hemi-	77.5	5.77	7.0	4.0	12.2		
J. W.	8	F	succin-	194.0	3.5	6.0	2.68	33.6		
H. C.	13	M	ate,	155.0	1.6	3.7	1.50	21.7		
B. E.	27	M	P.O.	233.0	7.0	14.1	4.50	58.8		
J. C.	28	F		233.0	9.1	15.0	4.00	39.5		
A. B.	36	M		233.0	9.3	16.9	4.10	56.8		
J. Z.	4	M		15.6	0	0.2	0.86	1.70		
H. Z.	6	M	S	15.6	0.1	0.35	2.05	3.53		
A. F.	8	F	hemi-	19.5	1.0	1.3	2.90	4.53		
R. B.	22	M	succ-	78.0	11.7	16.9	5.40	13.9		
A. B.	36	M	inate,	78.0	9.0	15.3	5.00	12.9		
R. E.	36	M	I.V.	78.0	9.6	10.8	5.18	11.1		
A. S.	5	M		22.5	1.45	1.76	0.26	0.96	4.4	4.4
J. F.	5	M		22.5	0.52	0.67	1.12	1.98	2.0	2.9
P. N.	9	F		58.0	2.90	3.70	2.30	3.90	1.47	2.3
E. O.	10	F	S	53.5	5.20	6.30	2.85	5.12	2.8	6.9
A. N.	11	M	acetate,	44.5	4.90	5.90	1.50	3.20	7.0	12.3
C. K.	24	M	P.O.	89.0	6.50	12.1	6.50	12.1	1.0	16.7
J. C.	27	F		89.0	9.50	13.7	2.30	7.30	5.0	50.0
B. E.	27	M		89.0	12.0	18.0	5.80	13.0	6.4	58.0
C. W.	28	M		89.0	11.0	19.7	8.40	16.9	5.0	50.4
A. B.	36	M		89.0	9.20	15.3	4.90	9.10	4.1	50.4
R. E.	36	M		89.0	8.10	9.70	4.80	7.10	4.8	7.6
B. E.	27	M	F§	90.0	12.0	11.3	5.10	28.5	2.4	16.3
C. W.	28	M	acetate,	90.0	11.1	12.0	8.40	31.6	4.8	20.1
A. B.	28	M	P.O.	90.0	10.9	9.90	4.70	17.3	3.9	21.6

* Control.
† After compound.
‡ S, "11-desoxycortisol."
§ F, hydrocortisone.

TABLE II

Per cent of dose excreted in urine

	Children						Adults					
	Subject	Age *yrs.*	Keto-steroids	Corti-coids	Formalde-hydogenic*	K/C†	Subject	Age *yrs.*	Keto-steroids	Corti-coids	Formalde-hydogenic	K/C
A‡	G. W.	5 mos.	0	10.8		0	B. E.	27	2.7	19.9		0.135
	S. M.	3	1.2	10.5		0.115	J. C.	28	2.5	14.8		0.167
	C. O.	6	1.7	10.5		0.160	A. B.	36	3.2	22.4		0.143
	J. W.	8	1.3	17.4		0.072						
	H. C.	13	1.4	14.0		0.097						
B§	J. Z.	4	1.0	5.0		0.200	R. B.	22	5.2	10.3		0.505
	H. Z.	6	1.0	9.5		0.105	A. B.	36	8.1	10.1		0.802
	A. F.	8	1.2	8.2		0.147	R. E.	36	1.6	7.6		0.215
C‖	A. S.	5	1.3	3.2	0	0.406	C. K.	24	6.3	7.4	17.7	0.852
	J. F.	5	0.6	3.9	4.0	0.166	J. C.	27	4.7	5.6	50.0	0.840
	P. N.	9	1.4	2.7	1.7	0.518	B. E.	27	6.9	8.1	58.0	0.852
	E. O.	10	2.1	4.2	7.7	0.500	C. W.	28	6.5	6.4	34.1	1.010
	A. N.	11	2.3	3.7	11.9	0.621	A. B.	36	6.9	4.7	52.0	1.465
							R. E.	36	1.8	2.6	3.1	0.692
S Average			1.3	8.0	5.1	0.239			4.7	10.0	35.8	0.640
F ¶							B. E.	27	0	26.0	15.6	
							C. W.	28	1.1	25.8	17.0	
							A. B.	36	0	14.2	18.8	

* Formaldehydogenic steroids (nonketonic).
† Ratio of per cent ketosteroids to per cent corticoids.
‡ S hemisuccinate P.O. (S is "11-desoxycortisol").
§ S hemisuccinate I.V.
‖ S acetate P.O.
¶ Hydrocortisone acetate P.O.

resents the relationship of the percentage dose converted to 17-ketosteroids over corticoids.

17-Ketosteroids

The rise in excretion of 17-ketosteroids, released by glucuronidase, was greater in the adults, particularly when the hemisuccinate of the steroid was given by vein, or the acetate given orally. In all experiments the adults demonstrated a greater conversion of Substance S to urinary 17-ketosteroids. A single exception among the adults, Subject R. E., exhibited less conversion than his age peers after intravenous hemisuccinate or oral acetate. His excretion of other steroid metabolites was also unique, as will be shown below. The difference between adults and children, in the increment of 17-ketosteroids, even when expressed as K/C ratio, was statistically significant ($p = 0.001$). As stated below, there are differences depending upon the form of Substance S; general averages are shown in Table II for statistical purposes only.

It has been previously demonstrated that the major 17-ketosteroid excreted after the administration of Substance S is etiocholanolone (*2*). Two collections of urine (Subjects A. B. and B. E., after the oral administration of the acetate) were pooled, and the total amount, minus the small aliquots employed for other tests, was hydrolyzed with beta-glucuronidase. After Girard separation, the residue was chromatographed on alumina for the resolution of the 17-ketosteroids. The total amount of 17-ketosteroids recovered from the column, as measured by the Zimmermann reaction on all fractions, was 21.9 mg. A single compound accounted for 11.9 mg. This proved to be etiocholanolone by infrared spectroscopy. The total quantity of etiocholanolone isolated from these two specimens agrees well with the total rise in 17-ketosteroids during the experiment, indicating that this compound accounts almost entirely for the increase; this also reveals that Substance S does not significantly suppress the 17-ketosteroids in urine.

Three experiments, in which adult subjects were given hydrocortisone acetate by mouth, are included in Table I for comparison. A rise of 17-

ketosteroid excretion was not noted, although this does not imply a failure of conversion to 17-ketosteroids. The extent of the conversion is partially obscured by the suppressive effect of hydrocortisone on endogenous 17-ketosteroid production.

The levels of 17-ketosteroids measurable after acid hydrolysis were approximately 10 per cent higher than after glucuronidase hydrolysis in all cases, and are not included in the tables.

Corticoids

The corticoids measured in the urine by the Porter-Silber reaction presumably represent 17,21-dihydroxy-20-ketosteroids. The greatest increment in corticoid excretion followed the administration of the hemisuccinate of Substance S. The rise following administration of the acetate of Substance S was considerably less than that after hydrocortisone acetate. Differences between adults and children in the excretion of corticoids were apparent, but these were relatively less than in the case of the 17-ketosteroids, as expressed in the K/C ratio in Table II.

The raised levels of urinary corticoids following the administration of Substance S were shown to reflect excretion of the reduced metabolite of this steroid, namely pregnane-3α,17α,21-triol-20-one ("tetrahydro S"). The total remaining volumes of urine from Subjects B. E. and A. B., after the oral administration of Substance S hemisuccinate, were hydrolyzed with beta-glucuronidase as outlined above. Column chromatography of the steroids by the method of Katzenellenbogen, Dobriner, and Kritchevsky (10) yielded two major fractions. The first was eluted with 3 to 4 per cent ethanol in methylene chloride and weighed 130 mg. This residue, after crystallization from methanol, melted at 186 to 187° C. and after acetylation, at 202 to 203° C. The acetylated compound was submitted to infrared examination, through the kindness of Doctor Thomas Gallagher, and was found to be identical with the diacetate of pregnane-3α,17α,21-triol-20-one ("tetrahydro S"). The second major fraction eluted is described below.

Formaldehydogenic steroids

The measurement of formaldehydogenic nonketonic steroids after the oral administration of S acetate was used as an index of the total reduction of Substance S to pregnane-3α,17α,20ξ,21-tetrol. Such metabolites of related C-21 steroids have been found in urine in significant amounts by Fukushima, Leeds, Bradlow, Kritchevsky, Stokem, and Gallagher (13). It is apparent that adults excrete greater quantities of this type metabolite after the administration of Substance S than after a dose of hydrocortisone. The conversion to the tetrol is greater in the adult than the child, after the administration of S acetate by mouth. Subject R. E., the adult who demonstrated a relatively low conversion of Substance S to 17-ketosteroids, also excreted smaller quantities of the C-20-reduced derivative.

Since such large quantities of the completely reduced derivative of Substance S were apparently excreted, as indicated by the formaldehydogenic method, the urine pools described under "Corticoids" were re-examined. During chromatography on silica, a large crystalline residue was obtained following elution with 6 per cent ethanol in methylene chloride. This material failed to give the blue tetrazolium or Porter-Silber reactions. The melting point of the free compound was 246 to 251° C., and after acetylation, 144 to 146° C. These properties were identical with those of a substance previously and tentatively identified by the present authors (14) as pregnane-3α,17α,20ξ,21-tetrol. Doctor Thomas Gallagher reported that by infrared examination this compound was virtually identical to P666, the material previously submitted.

DISCUSSION

These studies indicate that there are differences between the immature and adult human subject with respect to the metabolism of Substance S. In adults, there is a greater conversion of Substance S to 17-ketosteroids and metabolites reduced at C-20. It may be speculated that those systems responsible for the degradation or reduction of the side-chain are more efficient with the attainment of maturity; this would include many factors, such as hepatic flow and size, enzymes, cofactors and so forth. Further studies are in progress to determine whether similar differences exist in the metabolism of C-21 steroids other than Substance S. Furthermore, in spite of the differences shown in the metabolism of Substance S between children and adults, there is no evidence that

this steroid is normally released by the adrenal gland. Hence it cannot be specifically implied that this compound plays a definite role in the increase of 17-ketosteroids with increasing age. It is suggested that other similar precursors are responsible, and Substance S has been employed only as a model in these experiments.

The greater conversion of Substance S to 17-ketosteroids in adults suggests another explanation for the increased urinary excretion of 17-ketosteroids at adolescence. The greater facility with which the degradation of Substance S occurred in the older subjects indicates that, in some portion, the elevated 17-ketosteroids arise from C-21 precursors, although the latter compounds are not themselves produced in significantly greater amounts when related to the body surface area (1).

Although the excretion of corticoids has also been shown to be somewhat less in children, after the administration of the test compounds, the K/C ratio (Table II) clearly indicates that the differences in the 17-ketosteroids are considerably and significantly greater. One child (C. O., S hemisuccinate by mouth) revealed a ratio of 17-ketosteroids to corticoids similar to the adult. It is interesting that this subject was clinically more advanced than her years and her baseline excretion of 17-ketosteroids (5.7 mg. per day, Table I) was already approaching adolescent levels. Contrariwise, one adult (B. E.) consistently failed to show the degree of conversion to 17-ketosteroids or the reduction at C-20 noted in the other adults. This was regarded as a "normal variant," since the subject was in good health and the observation of such individuality in steroidal metabolism among normal subjects is not without precedent (15).

The major urinary products isolated after the administration of Substance S in these experiments included: etiocholanolone, tetrahydro-S and pregnane-3α,17α,20ξ,21-tetrol. Differences in results are noted between the various forms of Substance S and the route of administration. It has been reported by others (2) that the excretion of etiocholanolone is greatest after the oral administration of the acetate of Substance S. The data presented in three instances, for purposes of comparison, after the administration of hydrocortisone to adults are in agreement with other studies (16, 17). The excretion of 17-ketosteroids after the use of hydrocortisone does not lend itself to interpretation in these experiments. The precise identification of urinary 17-ketosteroids is required in order to arrive at the extent of conversion, when employing steroids which suppress endogenous adrenocortical function. However, there are larger amounts of urinary metabolites, reduced at C-20, after the administration of Substance S than with hydrocortisone. The recovery of cortol and cortolone, both highly water soluble, has not been checked; hence the difference may be artifactual. However, the order of the quantity measured as nonketonic formaldehydogenic steroids (Tables I and II) is reasonably close to that reported by isotopic techniques (13); and the inverse reciprocal relationships of the ketonic corticoids to the nonketonic steroids, between Substance S and Compound F reported here, are in keeping with a true difference in metabolism. Perhaps the presence of an oxygen function at C-11 handicaps the reduction at C-20 by biological systems.

The detailed chromatographic studies of some pools of urine in these experiments, in which three major metabolites of Substance S have been isolated and identified, show good agreement with the results indicated by standard routine analyses.

In a few subjects receiving Substance S hemisuccinate by mouth, fractional urine collections were obtained, for 8 and 16 hours after the dose. Sixty-five per cent of the total daily amount of 17-ketosteroids and corticoids was present in the first 8 hours. The investigations were not continued beyond 24 hours and the results do not indicate the total recoverable metabolites over several days. Therefore, the interpretation of differences is limited to the single 24 hour period, and the possibility that relatively larger quantities of metabolites might appear in the younger subjects over a prolonged period is unanswered. However, even if this were the case, significant differences in rates of metabolism would obtain during the first day.

SUMMARY

The administration of Substance S (11-desoxycortisol) to a group of human subjects revealed differences in the metabolism between adults and children. In mature subjects there was a greater

conversion of Substance S to 17-ketosteroids. The increment was shown to be primarily due to a rise in the urinary excretion of etiocholanolone. This suggests another mechanism for the rise in the urinary excretion of 17-ketosteroids at adolescence, apart from direct genesis of gonadal or adrenal C-19 steroids *per se*. In addition, the adults produced greater quantities of urinary metabolites reduced at C-20. It also appears that Substance S is more readily reduced at C-20 than hydrocortisone. Tetrahydro-S has been isolated and identified in the urine after the administration of Substance S; another compound has been isolated and tentatively identified as pregnane 3α,17α, 20ξ,21-tetrol.

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REFERENCES

1. Talbot, N. B., Zygmuntowicz, A. S., Wood, M., and Christo, E. Observations on adrenal cortical "sugar-fat-nitrogen" hormone ("11-17-OCS") and "17-ketosteroid precursor" production by normal and abnormal individuals of various ages *in* Proc. First Clinical ACTH Conference, J. R. Mote, Ed. Philadelphia, Blakiston & Co., 1950, p. 32.
2. Birke, G. The effect of Reichstein's Substance S on the excretion of 17-ketosteroids. Acta endocr. (Kbh.) 1954, **15**, 17.
3. Bongiovanni, A. M. A method adapted for the determination of urinary 17-ketosteroids in children. J. Pediat. 1951, **39**, 606.
4. Bongiovanni, A. M., Eberlein, W. R., and Thomas, P. Z. Use of an organic base in the Zimmermann reaction. J. clin. Endocr. 1957, **17**, 331.
5. Wilson, H. Chromogenic values of various ketosteroids in a micro modification of the Zimmermann reaction. Arch. Biochem. 1954, **52**, 217.
6. Silber, R. H., and Porter, C. C. Determination of 17,21-dihydroxy-20-ketosteroids in urine and plasma *in* Methods of Biochemical Analysis, D. Glick, Ed. New York, Interscience Publishers, Inc., 1957, vol. 4, p. 139.
7. Bongiovanni, A. M., and Eberlein, W. R. Critical analysis of methods for measurement of pregnane-3-alpha, 17-alpha, 20-alpha-triol in human urine. Analyt. Chem. 1958, **30**, 388.
8. Schneider, J. J. Further isolation of adrenocortical compounds from male urine. J. biol. Chem. 1952, **194**, 337.
9. Bongiovanni, A. M., and Eberlein, W. R. A microformaldehydogenic method for analysis of chromatographic fractions of adrenal steroids. J. Lab. clin. Med. 1956, **48**, 320.
10. Katzenellenbogen, E. R., Dobriner, K., and Kritchevsky, T. H. Separation of steroid hormones and their metabolites by partition type chromatography. J. biol. Chem. 1954, **207**, 315.
11. Lakshmanan, T. K., and Lieberman, S. An improved method of gradient elution chromatography and its application to the separation of urinary ketosteroids. Arch. Biochem. 1954, **53**, 258.
12. Pincus, G., and Pearlman, W. H. Fractionation of neutral urinary steroids. Endocrinology 1941, **29**, 413.
13. Fukushima, D. K., Leeds, N. S., Bradlow, H. L., Kritchevsky, T. H., Stokem, M. B., and Gallagher, T. F. The characterization of four new metabolites of adrenocortical hormones. J. biol. Chem. 1955, **212**, 449.
14. Eberlein, W. R., and Bongiovanni, A. M. Plasma and urinary corticosteroids in the hypertensive form of congenital adrenal hyperplasia. J. biol. Chem. 1956, **223**, 85.
15. Dobriner, K. Studies in steroid metabolism. XIX. The alpha-ketosteroid excretion pattern in normal males. J. clin. Invest. 1953, **32**, 940.
16. Brown, H., Willardson, D. G., Samuels, L. T., and Tyler, F. H. 17-Hydroxycorticosteroid metabolism in liver disease. J. clin. Invest. 1954, **33**, 1524.
17. Peterson, R. E., Pierce, C. E., Wyngaarden, J. B., Bunim, J. J., and Brodie, B. B. The physiological disposition and metabolic fate of cortisone in man. J. clin. Invest. 1957, **36**, 1301.

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THE ORIGIN OF PLASMA PROTEINS IN THE GUINEA PIG FETUS [1, 2]

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During intrauterine life the fetus receives generous supplies of nitrogen from the mother for the formation of tissue proteins. In Figure 1 are presented diagrammatically the theoretical precursors of fetal nitrogen, stressing the origin of fetal plasma proteins. Omitted from consideration are the simpler nitrogenous compounds such as urea and ammonia which are not likely to be important sources of nitrogen for tissue protein in the presence of adequate supplies of amino acids. The possible pathways (Figure 1, reading from bottom up) are as follows:

1. Circulating amino acids in the mother may be transferred across the placenta and synthesized into proteins by the fetus.
2. Circulating amino acids in the mother may be synthesized into plasma proteins by the placenta for use by the fetus.
3. Maternal plasma proteins may be degraded by the placenta and resynthesized by the fetus.
4. Maternal plasma proteins may cross the placenta intact.

Some evidence concerning routes 1 and 2 in the human have been previously presented (1). In this paper will be presented the results of an investigation into routes 1, 3 and 4. The guinea pig was chosen as the experimental animal because, similar to the human, antibodies are transferred from mother to fetus in high titer (2). It was therefore hoped that results concerning other plasma proteins would also be applicable to the human.[4] The data we have obtained in the present study with isotopically labeled materials indicate that maternal amino acids and maternal plasma proteins serve as significant precursors of fetal plasma proteins. The placenta, however, does not degrade maternal plasma proteins for this purpose.

METHODS

Preparation of isotopically labeled materials. S^{35}-labeled plasma proteins were prepared biosynthetically by injecting 200 microcuries of S^{35} methionine,[5] intraperitoneally, into a guinea pig, and repeating the injection in eight hours. Twelve hours later the guinea pig was bled by cardiac puncture, using heparin as an anticoagulant. The plasma was separated by centrifuging and was dialyzed against four changes of 2 liters of isotonic saline over a 48 hour period at 4° C. S^{35}-labeled chicken plasma was prepared in a similar fashion from a donor chicken.

To obtain plasma protein fractions for injection, the radioactive plasma proteins were separated by zone electrophoresis on starch or on polyvinyl chloride resin [6] (3). The fractions were eluted with isotonic saline and then dialyzed against saline to free the material of veronal. As a rule, 4 to 5 ml. of plasma was separated on a block. Pooling of fractions from more than one block was usually not done in order to avoid additional manipulation

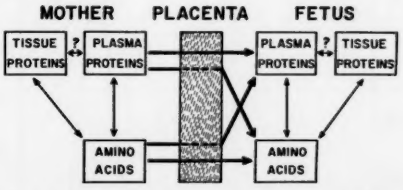

FIG. 1. DIAGRAMMATIC PRESENTATION OF POSSIBLE PRECURSORS OF FETAL PLASMA PROTEINS

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[2] The data have been presented in part at meetings of the Society for Pediatric Research, 1955 and 1956.

[3] John and Mary R. Markle Scholar in Medical Science.

[4] Brambell, Hemmings, and Henderson (2) have pointed out that this may be a superficial similarity. In an interesting series of experiments, they have demonstrated that the route of transfer of antibody protein in the guinea pig is not across the placenta but is across the splanchnopleure, a route that is anatomically unavailable to the human.

[5] Purchased from Abbott Laboratories, North Chicago, Ill.

[6] Geon Resin 420, Goodrich Chemical Company, Cleveland, Ohio.

and possibly damage of the proteins. If pooling was necessary, the pooled fractions were dialyzed against half-isotonic saline and then lyophilized to reduce to volumes that could be injected.

I^{131}-labeled plasma proteins were prepared according to the methods of Bauman, Rothschild, Yalow, and Berson (4) and of Pressman and Eisen (5).

Transfer experiments. Pregnant guinea pigs, estimated to be within two weeks of term, were used. Experiments were confined to this stage of pregnancy so that sufficient fetal blood could be obtained for analysis. The jugular vein of the mother was exposed surgically under Nembutal®-ether anesthesia and the injection made under direct vision. The amount of radioactivity injected varied with the material under study. For methionine, about 5 million counts per minute (cpm) with a specific activity of 20 million cpm per mg. was injected; total plasma, about 1 million cpm with a specific activity of about 20,000 cpm per mg.; plasma protein fractions, 100 to 300,000 cpm with the same specific activity. The fractions were in 15 ml. saline and were injected over a 20 minute period. Eighteen to 24 hours later a sample of maternal blood was obtained by cardiac puncture. The fetuses were delivered by hysterotomy and bled by decapitation.

In the experiments with radioiodinated proteins, the guinea pigs were given drinking water containing 0.3 N sodium iodide for 24 hours, and the injections were made with 2 ml. of 1.8 per cent sodium iodide in order to minimize utilization of liberated iodide. Seven $\times 10^6$ to 1.2×10^9 cpm of iodinated plasma proteins with specific activities ranging from 1.8×10^6 to 9×10^9 cpm per ml. was injected intravenously. The rest of the procedure was as described above.

Analytical methods. Maternal and fetal plasma proteins were separated by zone electrophoresis. The protein fractions were precipitated with equal volumes of 10 per cent trichloracetic acid (TCA) washed three times with 5 per cent TCA and redissolved in 0.05 N NaOH. An aliquot was taken for the determination of protein concentration by a modification of the method of Folin (6). For S^{35} determinations, the rest was transferred to planchettes. I^{131} radioactivity was determined in alkaline solution.

Determination of radioactivity. S^{35} radioactivity was determined in a D-46A Nuclear Flow Gas Counter, and the counts were corrected to infinite thinness. Many of the samples had little radioactivity. These were counted for one hour which provided a counting accuracy of at least plus or minus 10 per cent in those samples with observed counts of over 10 cpm over background. Those with less radioactivity were considered questionable and are indicated in the tables. I^{131} radioactivity was determined in a Tracerlab well-type scintillation counter, model SC 46.

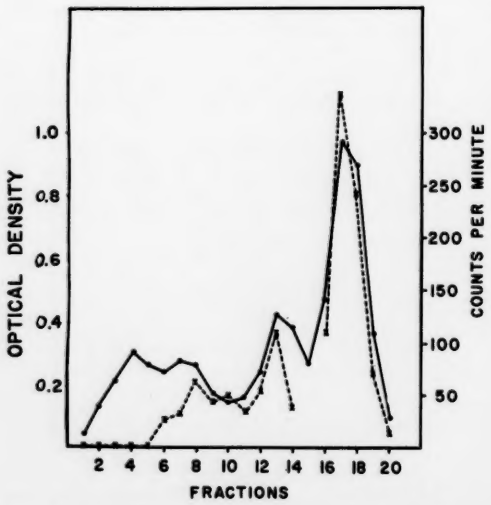

FIG. 2. INCORPORATION OF S^{35} METHIONINE INTO FETAL PLASMA PROTEINS

×----×, cpm per fraction; ●——●, protein concentration per fraction [the observed optical density after developing color according to method of Folin (6)].

RESULTS

Synthesis of plasma proteins by the guinea pig fetus

Guinea pig fetuses in the last week of gestation were delivered by hysterotomy and injected intraperitoneally with S^{35} methionine. Twenty-four hours later fetal blood was collected and the plasma proteins separated by zone electrophoresis. In Figure 2 are presented the protein curve and the radioactivity curve. There is no detectable incorporation of radioactivity into the gamma globulin fraction, although all other electrophoretic fractions appear to be synthesized by the fetus. This would indicate that the gamma globulin fraction is derived from either the placenta or the mother. Earlier investigations with human placenta (1) indicate that the placenta is an unlikely source.

The synthesis of plasma proteins by the fetus from amino acids transferred from the mother

S^{35} methionine was injected intravenously into pregnant guinea pigs and 24 hours later maternal and fetal blood samples were collected. Radioactivity was incorporated into all the plasma protein fractions in mother and fetus (Table I, A). The relatively high specific activities in the fetal plasma proteins are evidence of the rapid transfer of the amino acid and the efficient utilization by the

fetus. The notable exception is the fetal gamma globulin and the explanation probably lies in the inability of the fetus to synthesize this fraction. As a result the radioactivity must first be incorporated into maternal gamma globulin and then transferred to the fetus.

The transfer of S^{35}-labeled homologous plasma proteins across the placenta

The injection of S^{35}-labeled plasma proteins into the pregnant guinea pig resulted in the appearance of radioactivity in all the electrophoretic fractions in fetal plasma proteins (Table I, B). The results were very similar to those obtained by Whipple, Hill, Terry, Lucas, and Yuile in dogs with C^{14} lysine, although these investigators did not study the individual plasma protein fractions (7). The incorporation of radioactivity into fetal tissues in these experiments are not reported here but also agree well with those of Whipple and coworkers.

There still remains the possibility that the apparent transfer of intact plasma proteins actually represents degradation of the radioactive plasma proteins to smaller fragments which cross the placenta and are resynthesized by the fetus. This could be true of all fractions except the gamma globulin fraction. Although degradation of homologous plasma proteins by the placenta seems unlikely in view of the *in vitro* experiments reported below, this could result from maternal metabolism.

The transfer of heterologous plasma proteins labeled with S^{35} and I^{131}

The purpose of these experiments was to investigate the possibility indicated in the preceding paragraph. S^{35}-heterologous plasma proteins were used so that their fate could be followed both by radioactivity and by immunological methods. If the injected proteins were degraded and the radioactivity then incorporated into fetal plasma proteins, this would be readily detected immunologically.

S^{35} chicken plasma was injected into pregnant guinea pigs and 24 hours later maternal and fetal plasma were collected. Immunological identification of the radioactive proteins was made by preparing carrier precipitates in the plasmas with chicken plasma and an anti-chicken plasma (1) and then determining the amount of radioactivity incorporated in the specific precipitate (Table II). Most of this protein radioactivity (TCA-precipitable) in the maternal plasma was incorporated in the specific precipitate, but in the fetal plasma a considerable proportion was no longer identifiable immunologically as chicken plasma. Although these experiments were done with heterologous plasma proteins and might not be strictly applicable to those done with homologous plasma pro-

TABLE I

*Transfer of S^{35} methionine and plasma proteins from mother to fetus**

	Material injected	Maternal				Fetal			
		Albumin	Alpha	Beta	Gamma	Albumin	Alpha	Beta	Gamma
A	Methionine	161	297	367	156	59	217	183	20
		100	355	380	128	37	189	152	5
		146	312	436	230	63	225	190	26
B	Plasma proteins	81	80	100		9	13	11	25
		107	95	93	82	5	14	12	
		130	168	190	327	11	17	18	34
C	Albumin	240	15	3†	0	4	6	0	0
	Albumin	110	32	7	7†	4	3†	0	0
	Albumin	249	20	8†	4†	16	7	0	0
	Alpha	3	28	4	2	0	4	3†	0
	Beta-gamma	0	15	79	75	0	4†	2†	7
	Beta-gamma	0	6	46	135	0	0	21	17
	Gamma	0	0	29	59	0	0	0	6

* Specific activity in maternal and fetal plasma proteins in cpm per mg., 24 hours after injecting S^{35}-labeled materials into the mother.

† Calculated from less than 10 cpm above background.

TABLE II

Transfer of radioactive chicken plasma proteins across guinea pig placenta *

	Maternal		Fetal	
	TCA precipitate	Specific precipitate	TCA precipitate	Specific precipitate
S^{35} plasma proteins	14,850	10,820	950	150
	13,400	9,745	1,450	204
I^{131} plasma proteins	1,000,000	700,000	3,550	2,450
	1,100,000	965,000	4,000	3,850

* Twenty four hours after injection into the mother of the radioactive proteins, specific precipitates (chicken plasma plus anti-chicken plasma) were made in maternal and fetal plasma. Radioactivity incorporated in specific precipitates is compared with TCA-precipitable radioactivity (cpm per ml.).

teins, it was considered hazardous to interpret the results presented in the previous section as evidence for the transfer of intact plasma proteins.

Similar experiments with I^{131} chicken plasma produced significantly different results (Table II). Relatively less radioactivity was found in the fetal plasma protein, but the specific precipitate carried down the same proportion of the protein radioactivity in both maternal and fetal plasma. This indicated that the I^{131} proteins in the fetus were immunologically the same as those injected into the mother, and that the problem of degradation and reutilization would not confuse transfer experiments with this type of labeling. The results conform with the experience of investigators using isotopically-labeled plasma proteins for metabolic studies (8).

The transfer of S^{35}-labeled plasma protein fractions

In order to circumvent the problem of degradation and resynthesis and still use biosynthetically-labeled proteins, electrophoretically-separated fractions were injected. It was considered that if the pattern of radioactivity in the fetal plasma proteins differed from that obtained with S^{35} methionine, then the injected proteins were not degraded to the amino acid before reutilization. Evidence that the protein was transferred intact was obtained if the fetal fraction with the highest specific activity corresponded to that injected into the mother. The results are presented in Table I, C. Radioactivity was usually found in fractions adjacent to the one selected electrophoretically which we attributed to inaccuracies in the series of electrophoretic separations involved in preparation and analysis of samples.

When S^{35} methionine was injected into the mother, the lowest specific activity was found in the fetal gamma globulin, intermediate in the albumin, and highest in the alpha and beta globulin fractions. In those experiments in which S^{35} albumin and gamma globulin were injected, the specific activity in the respective fetal fractions were equal to or exceeded that in the alpha and beta fractions. This was considered satisfactory evidence that these proteins had been transferred to the fetus intact. The data for the alpha and beta globulins is not inconsistent with the same conclusion but is inadequate. The distribution of radioactivity in these experiments is not sufficiently different from that obtained with methionine.

Only a small fraction of the total number of experiments (60) done with S^{35}-labeled plasma proteins is presented in Table I. The reasons for failure were multiple: abortions, inadequate fetal plasma samples, insufficient radioactivity in the fetal samples, and a pattern of radioactivity in the fetal samples that could not be differentiated from that expected with degradation of maternal proteins and resynthesis by the fetus. In none of the rejected experiments did the data suggest transfer of other than the injected fraction. In one experiment alpha globulin was injected into the mother and the specific activity of the fetal gamma globulin greatly exceeded the maternal. It was evident that radioactive contamination had occurred and the results were discarded.

The transfer of I^{131}-labeled plasma proteins

I^{131}-labeled plasma proteins were resorted to so that smaller amounts of high activity protein could be used, and to further exclude the possibility of degradation and resynthesis. The experiments

TABLE III

Degradation of plasma proteins by tissue slices *

Tissue	TCA precipitate	TCA-soluble
1. Maternal liver	21,000	170
Fetal liver	21,000	240
Placenta	21,000	230
2. Maternal liver	20,000	114
Fetal liver	20,000	97
Placenta	20,000	83

* Radioactive plasma proteins (TCA-precipitable radioactivity) were incubated with tissue slices for four hours and the amount of degradation (TCA-soluble radioactivity) was measured.

with I^{131} chicken plasma (Table II) had already given immunological evidence that the latter did not occur in the course of transfer of radioactivity to the fetus. It is well recognized that iodination of proteins produces some alteration in the protein but useful metabolic information has been obtained from the technique (8). It seemed a reasonable assumption that qualitatively reliable results would be obtained.

The first iodination method used (4) extensively degraded the alpha and beta globulin fractions. The injection of plasma iodinated by this method yielded radioactivity in the albumin and gamma globulin fractions in mother and fetus (Figure 3). The almost exact reduplication of the maternal pattern of radioactivity in the fetus gave electrophoretic confirmation (in addition to the immunological evidence cited above) that I^{131} proteins were transferred intact, and that degradation with reutilization did not occur. Using the same iodination procedure, Rothschild and Oratz (9) have demonstrated the transfer of I^{131} albumin in the rabbit. When organic solvents were avoided in the iodination procedure (5) significant radioactivity was found in all plasma protein fractions in mother and fetus (Figure 4). There were differences in the amount of radioactivity in the individual fetal electrophoretic fractions, but it is not possible to conclude that this reflects differences in the efficiency of transfer. The very low activity in the fetal alpha globulins may reflect poor transfer, but it may merely indicate that this fraction is altered more extensively during iodination. It may be pertinent to this problem that I^{131} thyroxine which is bound to, and in effect labels, the alpha globulins without artificial iodination of the

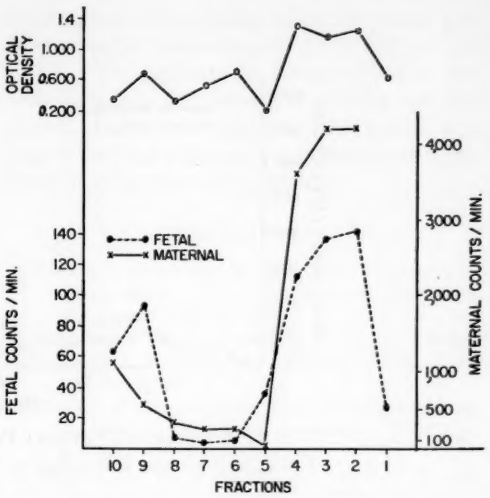

FIG. 3. TRANSFER OF I^{131} PLASMA PROTEINS ACROSS GUINEA PIG PLACENTA

The method of iodination in this experiment (4) apparently degraded the alpha and beta globulins. The top curve indicates protein concentration (6).

protein, is transferred in the rabbit from mother to fetus with about the same efficiency as albumin in the present study (10).

Degradation of maternal plasma proteins by placenta to provide precursors for fetal utilization

This possibility was investigated *in vitro* by incubating placental slices with S^{35}-labeled homologous plasma proteins. The extent of degradation was estimated by measuring the conversion of TCA-precipitable to TCA-soluble radioactivity. Slices of maternal and fetal liver were used as controls. The function of the liver is primarily synthetic (11).

Maternal and fetal liver, and placenta were obtained from pregnant guinea pigs under Nembutal®-ether anesthesia and dropped into chilled isotonic saline. Slices were prepared with a Stadie-Riggs slicer. The placental slices included both maternal and fetal surfaces. One gram of tissue was added to 2.5 ml. of Krebs-Ringer bicarbonate buffer (12) containing 10 μg. Aureomycin®, 5 mg. methionine, and radioactive plasma proteins in an amount sufficient to provide 20,000 cpm (3 to 6 mg.). The slices were incubated for four hours with constant shaking in a water

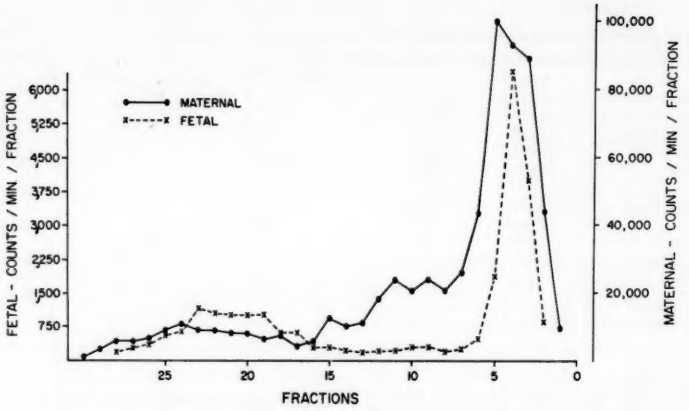

FIG. 4. TRANSFER OF I^{131} PLASMA PROTEINS ACROSS GUINEA PIG PLACENTA
Iodination of proteins as described by Pressman and Eisen (5).

bath at 37° C. At the end of incubation, the tissue slices were centrifuged down and discarded. Thirty-four mg. of methionine was added to the incubation medium and the proteins were precipitated with equal volumes of 10 per cent TCA. The supernatant was combusted and precipitated as barium sulfate, an aliquot of which was transfered to planchettes for determination of radioactivity (13). It is apparent from the table that the placenta does not demonstrate any specific capacity for the degradation of plasma proteins (Table III).

DISCUSSION

It is evident that maternal amino acids are transferred to the fetus where they are synthesized into plasma proteins. The fetal plasma proteins except for gamma globulin probably originate primarily in this way in the stage of pregnancy under investigation. The human fetal liver synthesizes plasma proteins efficiently as early as the third month of gestation (1) so that it is not unlikely that this is a major source of plasma proteins during most of pregnancy.

Evidence has also been presented for the transfer of maternal plasma proteins to the fetal circulation. The crucial experiments with S^{35}-labeled plasma proteins were those in which individual electrophoretic fractions were injected into the mother. It was only in this manner that the transfer of the intact protein could be differentiated from the transfer of radioactive fragments to the fetus with subsequent reincorporation into proteins. This approach proved technically difficult so that relatively few of the completed experiments were considered sufficiently satisfactory for presentation (Table I,C). Although the radioactivity in the fetal plasma in these experiments was low, it was sufficient to be determined with reasonable accuracy (an observed radioactivity of at least one and one-half times background). It is noteworthy that radioactivity of this magnitude was never found in a fetal fraction other than the one injected into the mother, or the immediately adjacent electrophoretic fraction. This was further indication that the observation was significant.

With I^{131} plasma proteins the two major disadvantages of the biosynthetically prepared plasma proteins were avoided. It was possible to inject trace amounts of plasma proteins of high specific activity, and the interpretation of results was not confused by reutilization of radioactive fragments. Extensive experience in metabolic experiments has demonstrated that metabolites containing the iodine label are not reincorporated into proteins (8). We have presented immunological and electrophoretic evidence that this is also true under the conditions of our experiments. On the other hand, the major advantage of the biosynthetically-labeled plasma proteins, that of close identity with the native proteins, can only be approximated with the radioiodinated proteins. Close approximation

can be achieved in metabolic experiments, but the same information is not available relative to permeability. Thus the results with I^{131} may be considered complementary to those with S^{35} plasma proteins. These have been presented in detail in the previous section. The conclusion from both series of experiments is that maternal plasma proteins are transferred intact to the fetus in relatively large amounts.

The question arises as to how much of the fetal requirements for plasma proteins could be met by transfer of plasma proteins from the mother. Two factors must be evaluated in the answer: the efficiency of transfer and the metabolic requirements of the fetus. The metabolic half-lives of different plasma proteins vary widely in the human, from a matter of hours for antihemophiliac globulin to weeks for albumin. If this also pertains to the guinea pig fetus it would require considerably different rates of transfer to satisfy the fetal demands for these two materials. There is no information concerning the rates of utilization of plasma proteins by the fetus. Another feature which limits the interpretation is the heterogeneity of the electrophoretic fractions that were studied (14). This could hide significant differences in transfer among proteins within a fraction. Certain general conclusions can be safely drawn. In agreement with previous work, gamma globulin appears to be derived entirely from maternal plasma. Using this as a basis of comparison, significant amounts of fetal albumin and beta globulin are exchanged with maternal plasma proteins. The data relative to the alpha globulins is inconclusive.

SUMMARY

1. The guinea pig fetus near term can synthesize all plasma proteins recognizable electrophoretically except for gamma globulin.

2. The fetus efficiently utilizes amino acids transferred from the maternal circulation for the synthesis of plasma proteins.

3. *In vitro* studies failed to demonstrate any specific proteolytic powers in the placenta which degrade maternal plasma proteins to make available for the fetus precursors for plasma protein synthesis.

4. The transfer of maternal plasma proteins to the fetus was studied using radioactive plasma proteins prepared biosynthetically (S^{35}) and *in vitro* (I^{131}). The problems in interpretation of data with each type of radioactive labeling are discussed. It was concluded that other plasma proteins in addition to the gamma globulins are transferred in large amounts to the fetus.

REFERENCES

1. Dancis, J., Braverman, N., and Lind, J. Plasma protein synthesis in the human fetus and placenta. J. clin. Invest. 1957, **36**, 398.
2. Brambell, F. W. R., Hemmings, W. A., and Henderson, M. Antibodies and Embryos. London, Athlone Press, 1951.
3. Kunkel, H. G., and Slater, R. J. Zone electrophoresis in a starch supporting medium. Proc. Soc. exp. Biol. (N. Y.) 1952, **80**, 42.
4. Bauman, A., Rothschild, M. A., Yalow, R. S., and Berson, S. A. Distribution and metabolism of I^{131}-labeled human serum albumin in congestive heart failure with and without proteinuria. J. clin. Invest. 1955, **34**, 1359.
5. Pressman, D., and Eisen, H. N. The zone of localization of antibodies. V. An attempt to saturate antibody-binding sites in mouse kidney. J. Immunol. 1950, **64**, 273.
6. Kunkel, H. G., and Tiselius, A. Electrophoresis of proteins on filter paper. J. gen. Physiol. 1951, **35**, 89.
7. Whipple, G. H., Hill, R. B., Jr., Terry, R., Lucas, F. V., and Yuile, C. L. The placenta and protein metabolism. Transfer studies using carbon^{14}-labeled proteins in dogs. J. exp. Med. 1955, **101**, 617.
8. The role of I^{131}-labeled proteins in biology and medicine. Ann. N. Y. Acad. Sci. 1957, **70**, 1–152.
9. Rothschild, M. A., and Oratz, M. Personal communication.
10. Hall, P. F., and Myant, N. B. Passage of exogenous thyroxine and of iodide between mother and foetus in pregnant rabbits. J. Physiol. 1956, **133**, 181.
11. Miller, L. L., and Bale, W. F. Synthesis of all plasma protein fractions except gamma globulins by the liver. The use of zone electrophoresis and lysine-E-C^{14} to define the plasma proteins synthesized by the isolated perfused liver. J. exp. Med. 1954, **99**, 125.
12. Umbreit, W. W., Burris, R. H., and Stauffer, J. F. Manometric techniques and tissue metabolism, 2nd ed. Minneapolis, Burgess Publishing Co., 1949.
13. Pirie, N. W. Studies in the sulphur metabolism of the dog. XI. Metabolism of methionine and related sulphides. Biochem. J. 1932, **26**, 2041.
14. Williams, C. A., Jr., and Grabar, P. Immunoelectrophoretic studies on serum proteins. I. The antigens of human serum. J. Immunol. 1955, **74**, 158.

INVESTIGATIONS ON THE MECHANISM OF THE LEUKOPENIC RESPONSE TO SHIGELLA ENDOTOXIN [1]

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A marked leukopenia, followed usually by a leukocytosis, is one of the constant reactions produced in experimental animals by the administration of endotoxin from Gram-negative bacilli. This reaction occurs along with other disturbances including fever, shock, hyperglycemia followed by hypoglycemia, thrombocytopenia and pathological changes in the gastrointestinal tract. The leukopenic response which has been described by many investigators (1–3) has been confirmed in this laboratory by our observations on dogs following the intravenous injection of Shigella endotoxin. We have also noted a rather marked leukopenia in some children with gastroenteritis due to Shigella (4).

In 1952, Penner and Klein (5) presented evidence that certain systemic effects, including the leukopenic response, following the intravenous injection of *Shigella dysenteriae* endotoxin are mediated by the direct action of this toxin on the central nervous system. From the results of cerebral cross-circulation experiments in which one of a pair of dogs received the entire blood supply to his brain from his partner, and vice versa, they reported that only the dog whose brain received its blood supply from the opposite dog, into whom the toxin had been injected, developed leukopenia and other systemic changes. The dog into which the toxin was injected failed to manifest the typical disturbances. They concluded from these experiments that the toxin acts directly on the brain, and suggested that the site of action was probably in the region of the hypothalamus.

Weil, MacLean, Spink, and Visscher (6) have recently presented evidence that does not support the idea that the shock produced by endotoxin is a result of a direct action on the central nervous system. They found that in cross-circulation experiments as described by Penner and Klein the shock and the leukopenia was not limited to the dog whose brain received the endotoxin. They further report that the shock is produced by endotoxin in dogs on whom chordotomy, with and without vagotomy, has been performed; and that arterial hypotension occurs in the decapitated dog given endotoxin.

The work of Mitchell and Stuart (7) on adrenalectomized cats suggests that the leukopenic response to Pyromen® is mediated through the adrenal gland. Soylemezoglu and Wells (8, 9), however, reported that the typical leukopenic response is obtained following the intravenous administration of pyrogen to adrenalectomized dogs.

The data to be presented here are the results of experiments designed to investigate the possible mechanisms of the leukopenic response to Shigella endotoxin with reference to the central nervous system and the adrenal gland.

MATERIAL AND METHODS

Endotoxin. "Crude endotoxin" was prepared from a culture of *S. flexneri* (type 2a) isolated from a patient with diarrhea in the pediatric ward of the University Hospital, and from a culture of *S. dysenteriae* (strain D_3) obtained from Dr. Sarah Branham (National Institutes of Health, Bethesda, Md). The toxins prepared from these two cultures are referred to as "J" toxin and "D_3" toxin, respectively. The "crude endotoxin" was prepared in the following manner: The bacteria were inoculated on brain heart infusion agar in Roux bottles and the growth harvested after 18 to 24 hours incubation by washing with normal saline. The growth from three bottles was then washed three times by centrifugation with normal saline and the sedimented cells were ground in a mortar for 30 minutes with alundum. Normal saline was added to make the total volume 100 ml. The suspension was centrifuged and the supernate was filtered (Seitz). The resulting filtrate constituted the "crude endotoxin" hereafter referred to as "toxin." It was not standardized, but by a series of trials it was found that the desired leukopenic response and the gastrointestinal lesions were invariably produced in dogs when 1 ml. per

[1] This investigation was supported by Grant No. E-1233 from the National Institutes of Health, Public Health Service, Department of Health, Education and Welfare.

kilogram of body weight was given intravenously. This dose was actually far in excess of the amount required to produce the typical responses but was used routinely unless otherwise stated.

The toxin was injected into either the femoral or brachial vein of both anesthetized (Nembutal®) and unanesthetized normal adult dogs, and blood samples were collected from either the femoral or brachial vein. The samples were collected in tubes to which a few heparin crystals had been added as an anticoagulant. White blood counts were performed in the conventional manner on a Spencer Bright Line counting chamber, using N/10 hydrochloric acid as the diluting fluid.

Cerebral cross-circulation was performed by the method described by Penner and Klein with the following modifications: The vertebral vessels were exposed by way of a low, anterior, mid-line, longitudinal, cervical incision and ligated before their entrance into the vertebral canal of the transverse process. The entire procedure of ligation of the vertebral vessels, cross-circulation and administration of the toxin, was done at one time, rather than the preliminary ligation of the vertebral vessels as described by Penner and Klein. With these exceptions, the procedure described by the above mentioned investigators was used. In two experiments human serum albumin labeled with radioactive phosphorus (P^{32}) was injected into Dog 1 at the same time as the toxin and radioactivity were measured in blood samples from both dogs at various intervals. A deep well counter was employed for these counts. A fall in temperature in the animals was prevented by the application of external heat, since it has been shown that a leukopenia will develop in dogs made hypothermic (10, 11).

Decapitation was performed at the level of the sixth cervical vertebra. Artificial respiration was maintained by means of a Stoelting respiration pump, and a fall in body temperature was prevented as above. Toxin was administered intravenously, and blood specimens were obtained at regular intervals.

Bilateral adrenalectomy was performed in a one stage procedure through a right subcostal incision. The adrenalectomized dogs were given 75 mg. of cortisone acetate (11 dehydro-17 hydroxycorticosterone-21 acetate) intramuscularly 24 hours prior to surgery and were maintained on this daily intramuscular dose until able to take the medication in oral form. They were then given a single daily dose of 75 mg. with additional sodium chloride in their drinking water for a period of 10 to 15 days following surgery. Twenty-four hours prior to the administration of the toxin oral cortisone acetate was omitted, and the animals received 25 mg. of the intramuscular preparation at this time. The animals appeared to be in good condition at the time of toxin administration but were in mild adrenal insufficiency as evidenced by altered serum electrolytes.

TABLE I

Leukopenic response to Shigella toxin in normal dogs

Time	Unanesthetized		Anesthetized	
	Dog 1	Dog 2	Dog 3	Dog 4
0 hour	10,300	5,500	15,000	10,000
1 hour	1,250	1,000	2,100	1,950
2 hours	1,200	3,300	2,900	5,000
3 hours	2,500	7,900	9,000	6,050
4 hours	5,500	8,750	16,000	12,400
5 hours	10,060	20,750	15,000	19,200

RESULTS

Normal dogs

In Table I the white blood counts in normal dogs, following the injection of Shigella toxin, are presented as examples of the leukopenic response. It will be noted that anesthesia did not alter this response. This typical leukopenia is a constant reaction to endotoxins.

TABLE II

White blood counts and radioactivity in cross-circulation dogs

Time	Experiment I W.B.C.		Experiment II W.B.C.		Experiment III				Experiment IV			
					W.B.C.		Counts/min.		W.B.C.		Counts/min.	
	Dog 1	Dog 2	Dog 1	Dog 2	Dog 1	Dog 2	Dog 1	Dog 2	Dog 1	Dog 2	Dog 1	Dog 2
After cross-circulation	19,000	12,400	13,100	10,000	10,500	6,050			12,500	13,000		
0 hour	Toxin into Dog 1								Toxin and radioactive albumin into Dog 1			
5 min.											3,045	745
30 min.	3,100	7,000	2,200	2,200	6,300	3,800	5,642	4,868	4,500	7,000	2,281	1,537
60 min.	3,600	4,800	2,300	1,350	2,200	4,600			4,600	3,000		
80 min.	3,300	2,250										
90 min.	4,900	2,250	4,600	3,250	6,400	9,700			4,000	3,700		
120 min.					3,800	7,000			3,800	3,300		
150 min.					3,100	6,000			3,300	1,450		
180 min.					9,500	18,000	4,339	3,997	3,850	9,200	1,709	1,607

TABLE III

Leukocyte response to Shigella toxin in decapitated dogs

Time	Controls—no toxin				"J" toxin		D_2 toxin		
	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Dog 6	Dog 7	Dog 8	Dog 9
Pre-op.	13,500	21,300		15,000	13,050		6,050	13,000	14,000
Post-op.	17,250	35,000	40,250	11,000	14,500	12,200	11,200	25,000	14,450
0 hour						Toxin, intravenously			
15 min.	14,800	22,000	33,150	13,200	900	5,850	2,160	1,700	8,000
30 min.	13,700	25,000	28,850	12,000	1,700	2,400	2,250	6,000	950
45 min.				15,000	1,500		2,250	1,650	850
60 min.	15,200	33,000		17,000	1,550	6,250	2,800	1,400	1,500
75 min.				15,000	1,850		2,300	1,050	
90 min.	11,200			12,000	3,000		2,600		
105 min.				14,000	2,700		2,600		
120 min.	11,900			11,800		5,000	2,550		

Cerebral cross-circulation

The white blood counts in the paired dogs of the cross-circulation experiments are presented in Table II. "J" toxin was used in Experiments I and II and "D_2" toxin in Experiments III and IV. In contrast to the results obtained by Penner and Klein, a definite leukopenia developed in both dogs of each pair. The fact that the desired separation of the circulations in the two dogs was not obtained by the procedure used is obvious on examination of the radioactive counts in the blood samples of the paired dogs in Experiments III and IV. Five minutes after injection of the labeled albumin into Dog 1 in Experiment IV, the activity of the blood in Dog 2 was approximately 24 per cent of the blood from Dog 1; after 30 minutes it was between 65 and 85 per cent. At the end of three hours there was complete equilibration of the radioactive material in the circulations of the two dogs. Postmortem examination of the gastrointestinal tracts of the paired dogs revealed no differences in the lesions present in the two animals.

TABLE IV

Leukopenic response to Shigella toxin in adrenalectomized dogs

Time	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Dog 6
0 hour	17,900	12,000	11,000	42,000	30,000	19,000
15 min.	5,300	8,200	2,850		5,000	9,050
30 min.	*	2,100	5,100	7,000	1,600	2,800
45 min.		1,600	2,000	4,500	2,900	2,850
60 min.		950	1,200	4,300	*	3,850
75 min.			10,600	6,000		15,050
90 min.		550	9,300	8,800		10,700
105 min.			*			7,200
120 min.		3,000		8,200		7,400
135 min.				*		10,000
150 min.		2,300				12,500
165 min.		*				20,000
						*

* Dog expired.

Decapitation

The results of the white blood counts in the decapitated animals are tabulated in Table III. It may be noted that in the control animals no significant leukopenia developed after decapitation. This is in marked contrast to the definite leukopenic response in the decapitated animals which received the toxin. This response is graphically presented in Figure 1 which shows the per cent change in the leukocyte count of the decapitated dogs. In the control animals the greatest fall in the white blood count was 36 per cent, whereas in all the animals which received toxin there was a drop of at least 80 per cent within 30 minutes after the injection of the toxin. As in the previous experiment, toxin from both *S. flexneri* and *S. dysenteriae* was used.

Postmortem examination of the gastrointestinal tracts of the decapitated dogs revealed severe hemorrhagic lesions, indistinguishable both grossly and microscopically in the control and toxin-injected animals.

Adrenalectomy

The leukocyte response to Shigella toxin in adrenalectomized dogs is shown in Table IV. Again it will be noted that these animals responded to the toxin by the development of a definite leukopenia. The dose of toxin administered to Dog 1 was 0.5 ml. per kilogram of body weight, and the animal expired 20 minutes later. Subse-

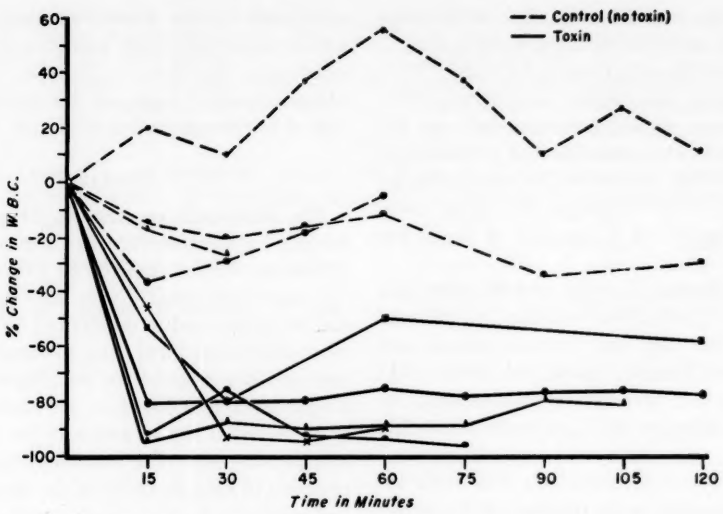

FIG. 1. PER CENT CHANGE IN LEUKOCYTE COUNT IN DECAPITATED DOGS FOLLOWING THE INTRAVENOUS ADMINISTRATION OF SHIGELLA TOXIN

quently, the dose of toxin employed was 0.1 ml. per kilogram of body weight, and with this dose death occurred within three hours.

Postmortem examination of the gastrointestinal tracts in these animals revealed hemorrhagic lesions as in the other experiments.

DISCUSSION

While the results of the cross-circulation experiments presented here are quite different from those of Penner and Klein (5), they are in agreement with the results reported by Weil and coworkers (6). The latter investigators concluded that this technique did not satisfactorily exclude the anastomotic channels in the skin, muscle and within the spinal canal, and, therefore, the endotoxin entered the opposite circulation. They note the fact that the endotoxin used by Penner and Klein was produced from *S. dysenteriae* which is known to differ from other Gram-negative bacilli in that it produces a neurotoxin which acts as an exotoxin. In our experiments this variable was eliminated, since toxin from a culture of *S. dysenteriae,* known to produce a potent neurotoxin, as well as toxin from a culture of *S. flexneri* which does not produce a neurotoxin was used. As in the experiments of Penner and Klein, and Weil and associates, an excess of toxin was employed in our studies.

Although separation of the circulations was not achieved in the cross-circulation experiments, the results of the decapitation studies would exclude the brain as essential for the production of the leukopenic response. The possibility exists that there is more than one mechanism for the production of the leukopenia and that one of these does involve the brain. To disprove this possibility it would be necessary to obtain a satisfactory separation of the two circulations, and this has not been done in the experiments here reported. This problem might be answered in spite of the incomplete separation of the circulation by adjusting the dose of toxin so that the minimum neutropenic quantities of the toxin were injected into Dog 1. If a leukopenia occurred in Dog 2 under these circumstances it would be expected that the cause was central stimulation. Attempts to establish the desired minimum dose of toxin have been very discouraging due to the rather marked variation of response in individual dogs to small doses of toxin. Although the possibility of a second mechanism which does involve the brain has not been disproved, it is difficult to explain the failure of development of leukopenia in Dog 1 in the experi-

ments of Penner and Klein in view of the large doses of toxin employed by these investigators.

The studies of Braude, Carey, and Zalesky (12) are of interest in this regard, in that they were unable to detect significant radioactivity in the brains of rabbits which had received *E. coli* endotoxin labeled with radioactive sodium chromate. They did find a large amount of the labeled endotoxin in the buffy coat at the time of the leukopenia.

Chambers, Koenig, Koenig, and Windle (13) have reported a leukopenic response to bacterial pyrogens in the dog after cervical spinal cord transection, and Pásztor, Lissák, and Martin (14) found that the cat, after total sympathectomy, responds to the injection of *E. coli* endotoxin with a leukopenia.

Delaunay (1) has attributed the leukopenic response to endotoxins to the retention of the leukocytes in the capillaries of the lungs, liver and spleen. The ultimate fate of these leukocytes—that is, whether they are destroyed or reintroduced into the circulation—is unknown. The data presented here, it would appear to us, exclude the brain as an essential site of action of the endotoxin in the production of a leukopenia.

No conclusions can be drawn as to the action of Shigella toxin on the central nervous system in the pathogenesis of the gastrointestinal lesions, since the decapitation procedure produced lesions indistinguishable from those resulting from the toxin alone. Keller (15), in 1936, described the production of hemorrhagic lesions in the digestive tract by intracranial trauma.

Thomas (3) has commented on the increased susceptibility of adrenalectomized animals to endotoxin. This fact was confirmed by our observations on adrenalectomized dogs as evidenced by the reduced dose of toxin necessary to produce death. Lewis and Page (16) have reported that adrenal extract and steroids exert a protective power against bacterial toxins in adrenalectomized rats. They found a decrease in the total leukocyte count in adrenalectomized rats two hours after the administration of typhoid vaccine, provided the rats had been maintained on saline alone. If, however, the adrenalectomized animals were treated with adrenal extract or compound A, there was no significant change in the total white blood count at the same period of time following the administration of the typhoid vaccine. Our observations show that there was no alteration in the leukopenic response to Shigella toxin in the adrenalectomized dogs and thus confirm the findings of Soylemezoglu and Wells (8, 9).

SUMMARY

The leukopenic response to Shigella toxin in the dog has been confirmed. The cerebral cross-circulation studies reported by Penner and Klein (5) have been repeated with some modification in the technique, and a satisfactory isolation of the two circulations was not obtained. The decapitated dog responds to the Shigella toxin with a leukopenia, and therefore, we exclude the brain as an essential site of action of the toxin in this specific response. The adrenalectomized dog also responds to Shigella toxin by the development of a leukopenia.

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REFERENCES

1. Delaunay, A. Mise en évidence d'une nouvelle propriété des antigènes glucido-lipidiques: Leur pouvoir leucopénisant. C. R. Soc. biol. (Paris) 1943, **137**, 589.
2. Bennett, I. L., Jr., and Beeson, P. B. Properties and biologic effects of bacterial pyrogens. Medicine 1950, **29**, 365.
3. Thomas, L. The physiological disturbances produced by endotoxins. Ann. Rev. Physiol. 1954, **16**, 467.
4. Donald, W. D. Unpublished data.
5. Penner, A., and Klein, S. H. The pathogenesis of experimental dysentery intoxication. J. exp. Med. 1952, **96**, 59.
6. Weil, M. H., MacLean, L. D., Spink, W. W., and Visscher, M. B. Investigations on the role of the central nervous system in shock produced by endotoxin from Gram-negative microorganisms. J. Lab. clin. Med. 1956, **48**, 661.
7. Mitchell, S. Q., and Stuart, E. G. Role of adrenal gland in mechanism of leucocyte changes upon administration of Pyromen (abstract). Amer. J. Physiol. 1951, **167**, 810.
8. Soylemezoglu, B., and Wells, J. A. Comparison of leucocyte response to ACTH and bacterial pyrogen. Proc. Soc. exp. Biol. (N. Y.) 1951, **77**, 43.
9. Soylemezoglu, B., and Wells, J. A. Studies on the mechanism of the leucocyte response to bacterial pyrogen. J. Pharmacol. exp. Ther. 1951, **101**, 33.

10. Villalobos, T. J., Adelson, E., and Barila, T. G. Hematologic changes in hypothermic dogs. Proc. Soc. exp. Biol. (N. Y.) 1955, **89**, 192.
11. Helmsworth, J. A., Stiles, W. J., and Elstun, W. Leukopenic and thrombocytopenic effect of hypothermia in dogs. Proc. Soc. exp. Biol. (N. Y) 1955, **90**, 474.
12. Braude, A. I., Carey, F. J., and Zalesky, M. Studies with radioactive endotoxin. II. Correlation of physiologic effects with distribution of radioactivity in rabbits injected with lethal doses of *E. coli* endotoxin labelled with radioactive sodium chromate. J. clin. Invest. 1955, **34**, 858.
13. Chambers, W. W., Koenig, H., Koenig, R., and Windle, W. F. Site of action in the central nervous system of a bacterial pyrogen. Amer. J. Physiol. 1949, **159**, 209.
14. Pásztor, J., Lissák, K., and Martin, J. Untersuchungen am Blutbild total sympathektomierter Katzen. Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak. 1942, **199**, 228.
15. Keller, A. D. Ulceration in the digestive tract of the dog following intracranial procedures. Arch. Path. (Chicago) 1936, **21**, 127.
16. Lewis, L. A., and Page, I. H. Studies on the protective power of adrenal extract and steroids against bacterial toxins in adrenalectomized rats. Ann. N. Y. Acad. Sci. 1949, **50**, 547.

EFFECT OF BLOOD PRESSURE ALTERATIONS ON INTRARENAL RED CELL–PLASMA SEPARATION [1, 2]

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Recent reports have provided evidence for the separation of red cells and plasma within the kidney (1–3). Pappenheimer and Kinter have proposed an ingenious theory of plasma skimming to explain this phenomenon (2). According to these authors the energy for the separation is derived from the arterial pressure. Hence, there should be less separation at low pressure, and more at higher pressure. To test the validity of this hypothesis, simultaneous red cell and plasma transit times through the kidney were measured at normal and at low arterial pressures in 12 mongrel dogs.

METHODS

Dogs weighing 8 to 12 Kg. were anesthetized with sodium pentobarbital, 25 mg. per Kg. intravenously. The left kidney was exposed and the renal artery and vein dissected free. Renal nerves were sectioned in all cases. A ligature was placed around the renal artery and tightened for a few seconds. During this time, the renal vein was incised and a polyethylene catheter (2.5 mm. inside diameter) was tied into the renal segment. The free end of the catheter was rapidly connected to a similar catheter that had previously been inserted in a femoral vein. When the renal artery occlusion was released, blood was seen to surge out of the kidney into the femoral vein. Flow through the catheters was unrestricted, all connections having internal diameters the same as the polyethylene tubing. In effect, the procedure had established a long exteriorized renal vein (Figure 1).

In all experiments aortic pressure was continuously monitored using a strain-gage transducer and direct-writing oscillograph.

Five ml. of a saline suspension of the dogs' red cells previously labeled with 100 microcuries of Cr^{51}, and washed free of any extracorpuscular Cr^{51} activity, was mixed with 20 microcuries of I^{131} tagged human serum albumin. One-half ml. of this mixture was injected into the renal artery over a period of 2 to 5 seconds. Just prior to injection, the renal venous catheter was disconnected from its coupling to the femoral venous catheter and permitted to drain freely. From the start of injection until 60 seconds later, total renal blood flow was continuously collected at 2 or 5 second intervals into paraffined test tubes containing dried heparin solution.

Seven of the animals were then bled from the femoral artery until the blood pressure was reduced and stabilized at about one-half of the control level. During the bleeding and stabilization period, the renal venous catheter was reconnected to the femoral vein. The renal arterial injection of isotope mixture was then repeated with total renal venous blood collection at the low arterial pressure for 120 seconds. The blood pressure frequently fell further or rose slightly at the start of this collection period. However, only those cases in which the pressure was constant after the isotopes were injected are reported. In two additional experiments, hydralazine (1 mg. per Kg.) was used to lower the blood pressure after the control measurement, and in one experiment, norepinephrine was continuously infused to raise the pressure after the control run. In two other experiments good control results were obtained, but as a result of technical difficulties low pressure curves were not obtained. However, these cases are included in the control series. In one further experiment the control blood samples were inadvertently contaminated with radioisotopes, but the low pressure (posthemorrhage) data are included.

Following collection of the samples, 0.5 ml. aliquots of whole blood were pipetted into glass vials. These were centrifuged and the red cells were washed three times with normal saline solution. The Cr^{51} activity of these washed aliquots was counted in a well-type scintillation counter. Their activity was expressed as Cr^{51} counts per ml. of whole blood.

The remaining blood in the collection tubes was centrifuged, and 0.5 ml. aliquots of the supernatent plasma were counted in a similar manner. Plasma activity was expressed as I^{131} counts per ml. whole blood, after correcting for the hematocrit obtained by centrifugation of at least three representative specimens (2,000 × G for 60 minutes).

From the Cr^{51} counts per ml. in each sample, and its time of collection (corrected for delay in the collecting system catheter and using the midpoint of the injection period as zero time), the mean circulation time of the

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[2] Supported in part by the United States Public Health Service Grant H-2987 (National Heart Institute).

[3] Research Fellow, American Heart Association.

[4] Work done in part during the tenure of an Established Investigatorship of the American Heart Association.

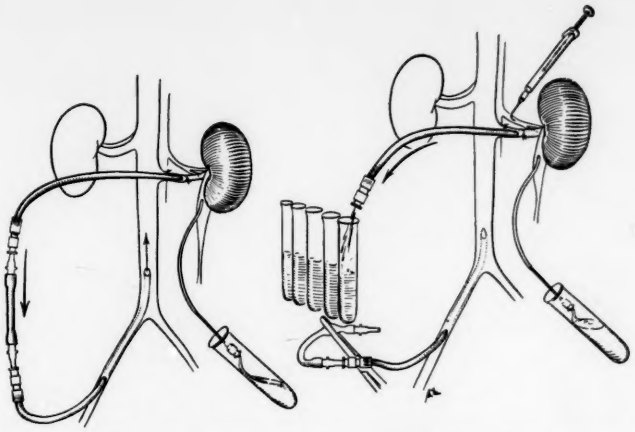

FIG. 1. DIAGRAM OF PROCEDURE USED TO OBTAIN SAMPLES FOR TRANSIT TIME CALCULATIONS

red cells was determined. Similarly, from the I^{131} albumin counts the plasma mean circulation time was calculated. Curves were integrated to the time where the concentration had fallen to less than 1 per cent of the peak value (Figures 2 and 3).

Intrarenal hematocrits were calculated from the formula

$$HCT_{KID.} = \frac{\frac{Tr}{Tp}}{\frac{Tr}{Tp} + \frac{1 - HCT_{L.V.}}{HCT_{L.V.}}},$$

where $HCT_{KID.}$ and $HCT_{L.V.}$ are the symbols for intrarenal hematocrit and arterial hematocrit, and Tr and Tp are the mean transit times of red cells and plasma, respectively (3).

Renal resistance was determined from the recorded pressure and measured flow. The peripheral resistance unit (PRU) was defined as the arterial pressure in mm. Hg divided by the flow in ml. per second.

RESULTS

Mean circulation time

In all studies the mean transit time for red cells was slightly shorter than that of the plasma. In the control group the ratio of red cell to plasma transit times averaged 0.82 ± 0.06 (S.D.) (Table I). In the group of dogs whose blood pressure

TABLE I

Red cell and plasma (albumin) mean transit time at normal arterial pressure

Dog	Pressure	Flow	Resistance	Tr*	Tp†	Tr/Tp	$HCT_{L.V.}$	$HCT_{KID.}$‡	$HCT_{KID.}/HCT_{L.V.}$
no.	*mm. Hg*	*ml./sec.*	*PRU*	*sec.*	*sec.*		%	%	
0830	125	1.70	74	8.4	9.9	0.85	50.0	45.9	0.92
1002	90	0.81	111	9.2	10.7	0.86	43.3	39.2	0.91
1017	130	1.10	118	5.9	6.7	0.88	40.5	37.3	0.92
1121	96	1.00	96	7.5	9.2	0.82	52.0	47.1	0.91
1128	100	1.38	72	9.1	11.1	0.82	52.9	47.9	0.91
1205	125	1.47	85	1.9	2.9	0.66	45.5	35.8	0.79
1101	125	1.00	125	8.1	9.6	0.84	28.2	24.8	0.88
1024	135	1.04	130	9.3	11.7	0.79	48.4	42.6	0.88
0208	125	1.50	83	7.5	8.8	0.85	42.9	35.3	0.82
0415	110	1.06	104	8.3	10.1	0.82	45.0	40.2	0.89
0416	110	2.00	55	4.9	5.9	0.83	51.3	46.6	0.91
Average		1.28				0.82	45.5	40.2	0.89
S.D.						±0.06			±0.04

* Tr, mean transit time of red cells.
† Tp, mean transit time of plasma (albumin).
‡ $HCT_{KID.}$ is calculated from formula given in text (see reference 3).

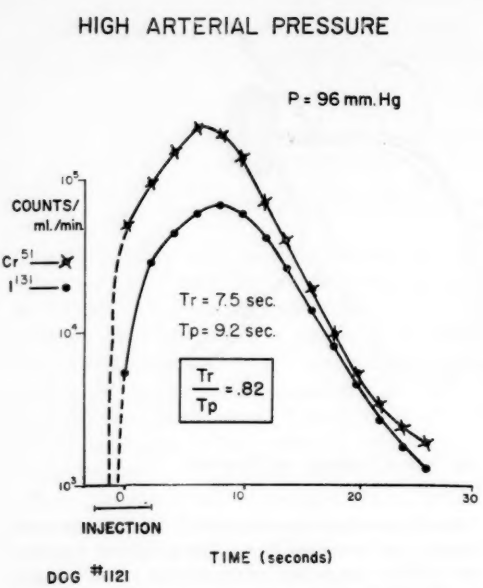

FIG. 2. TIME-CONCENTRATION CURVES OBTAINED FOLLOWING INJECTION OF Cr^{51} LABELED RED CELLS AND I^{131} LABELED ALBUMIN INTO THE RENAL ARTERY

Zero time is taken as the midpoint of the injection period. Concentrations of samples are plotted at the midpoint of their collection period minus the catheter delay time. Catheter delay time was calculated from the volume of the collecting system in ml. divided by the flow rate in ml. per second. The ratio of transit times is 0.82.

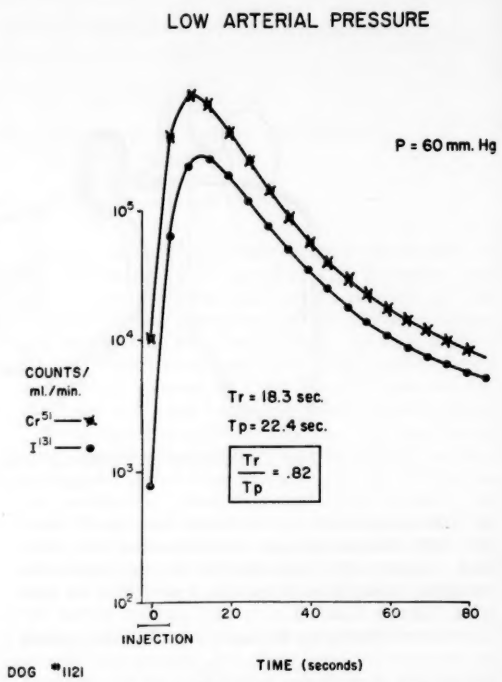

FIG. 3. TIME-CONCENTRATION CURVE FROM THE SAME KIDNEY AS IN FIGURE 2 FOLLOWING REDUCTION IN ARTERIAL BLOOD PRESSURE BY BLEEDING

Note the transit time ratio remains 0.82.

was lowered by hemorrhage this ratio averaged 0.79 ± 0.06 (Table II). Following lowering of the arterial pressure with hydralazine, the transit time ratios were 0.88 and 0.72 in the two animals studied. In the dog whose blood pressure was elevated by norepinephrine infusion the ratio was 0.88 (Table II).

Intrarenal hematocrit

The calculated intrarenal hematocrit in the control group was found to average 89 ± 4 per cent of the arterial hematocrit (Table I). This was not significantly altered by hemorrhage (86 ± 4 per cent) or by hydralazine (93 and 82 per cent) (Table II). Norepinephrine raised the arterial hematocrit from 51.3 to 56.0 but the ratio of calculated intrarenal to large vessel hematocrit was not significantly altered (0.91, control; 0.94 after norepinephrine) (Tables I and II).

Renal resistance

In the six animals in whom control and post-hemorrhage runs are reported, the renal resistance (P/F) rose from an average of 93 PRU to 146 PRU (Tables I and II). In spite of the rise, no significant alterations occurred in intrarenal hematocrit. In the hydralazine treated dogs, there was a fall in calculated resistance but no significant change in intrarenal hematocrit. In the norepinephrine infused animal the renal resistance rose markedly from 55 to 825 PRU but the transit times ratio and hematocrit ratio were not significantly altered.

DISCUSSION

The method of sample collection and catheter delay correction could result in an error in mean

TABLE II

Red cell and plasma (albumin) mean transit time after pressure alterations

Dog	Pressure	Flow	Resistance	Tr	Tp	Tr/Tp	HCTL.V.	HCTKID.	HCTKID./ HCTL.V.	Method of altering B.P.
no.	*mm. Hg*	*ml./sec.*	*PRU*	*sec.*	*sec.*		%	%		
0830	90	0.46	196	31.1	36.6	0.85	54.4	45.5	0.84	Hemorrhage
1002	70	0.29	241	21.4	25.9	0.83	42.4	37.9	0.89	Hemorrhage
1017	30	0.36	83	15.2	19.8	0.77	36.0	30.2	0.84	Hemorrhage
1121	60	0.49	122	18.3	22.4	0.82	54.1	49.1	0.91	Hemorrhage
1128	45	0.47	96	29.3	37.5	0.78	53.0	46.7	0.88	Hemorrhage
1205	30	0.22	136	10.5	16.0	0.66	45.5	35.5	0.78	Hemorrhage
0920	70	0.23	304	17.0	20.8	0.82	37.6	33.1	0.88	Hemorrhage
Average						0.79	46.1	39.7	0.86	
S.D.						±0.06			±0.04	
0208	70	1.33	53	8.3	9.4	0.88	44.0	40.9	0.93	Hydralazine
0415	50	0.52	96	10.5	14.5	0.72	45.0	37.1	0.82	Hydralazine
0416	165	0.20	825	10.0	11.4	0.88	56.0	52.7	0.94	Norepinephrine

transit time determinations as large as one second, but would affect the red cell and albumin data identically. For this reason, no attempt was made to calculate the intrarenal blood volume from the transit time data. However, errors of one second affecting both red cells and albumin would not produce significant changes in the *ratio* of transit times at the ranges found in this study.

The plasma skimming theory of Pappenheimer and Kinter (2) predicts a change in the ratio of red cell to plasma transit times and a rise in intrarenal red cell concentration with alterations in blood pressure. In spite of marked reductions in velocity of blood flow through these kidneys from an average of 1.3 ml. per second at normal pressures to 0.36 ml. per second at low pressure following hemorrhage, the *transit time ratio* was not significantly changed. In addition, no rise in calculated intrarenal hematocrit was observed. Similarly, hydralazine or norepinephrine, though markedly affecting blood pressure, did not alter the transit time relationships significantly. Thus it appears that the intrarenal hematocrit is independent of blood pressure (Figure 4). Almost

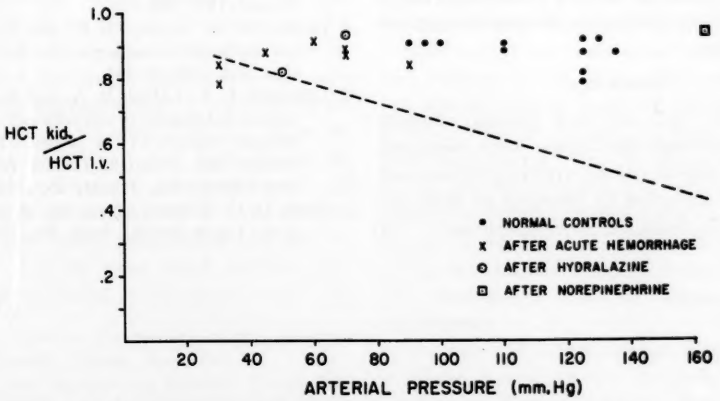

FIG. 4. EFFECT OF ARTERIAL BLOOD PRESSURE ON HEMATOCRIT

Ordinate, ratio of intrarenal hematocrit (HTC_{KID}) to large vessel hematocrit ($HCT_{L.V.}$); abscissa, arterial blood pressure during sample collection period. The dotted line would be the relationship expected from the hypothesis proposed by Pappenheimer and Kinter (2).

identical results have been reported by Ochwadt in studies with isolated blood-perfused kidneys (4). The changes in renal resistance that occurred following hemorrhage or drug administration must have resulted from vascular tone changes, rather than viscosity alterations due to intrarenal red cell concentration changes.

The transit times for red cells are somewhat less than that for plasma albumin at both high and low blood velocity. This observation argues against axial streaming or plasma skimming as the mechanism for the intrarenal red cell shunt. Some anatomic arrangement in the kidney must permit some red cells to traverse shorter paths than albumin entering at the same time. It is suggested that a long circuit pathway exists that permits mainly plasma or albumin to enter, and excludes red cells because of a very small entrance diameter. Recent observations reported from this laboratory provide evidence for an extravascular albumin space in the kidney which is rapidly equilibrated with the blood plasma (5, 6). Electron microscopic studies of the vascular bed of the rat kidney have demonstrated that both glomerular and peritubular capillaries are fenestrated with pores capable of permitting the passage of albumin (7). It seems likely that the long circuit albumin pathways are in reality part of the extravascular spaces in the kidney. The true intravascular hematocrit of the kidney may well be very close to that of the arterial blood. Further studies employing macromolecules as plasma indicators are now in progress to determine this.

SUMMARY

Simultaneous red cell and plasma albumin transit times through the kidney were measured in 12 mongrel dogs at normal arterial pressure and after pressure alteration by bleeding or drug administration (hydralazine, norepinephrine). In spite of marked changes in velocity of blood flow, no significant alterations in relative red cell to plasma transit times were observed. Calculated intrarenal hematocrits before or after acute bleeding were almost identical, and averaged 88 per cent of the arterial hematocrit. This study fails to support the plasma skimming theory of renal autoregulation. The evidence suggests the existence of long circuit plasma or albumin paths through the kidney which, at either normal or low pressure and velocity of flow, are too narrow at their entrance to permit passage of red cells.

ACKNOWLEDGMENT

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REFERENCES

1. Allen, T. H., and Reeve, E. B. Distribution of "extra plasma" in the blood of some tissues in the dog as measured with P^{32} and T-1824. Amer. J. Physiol. 1953, **175**, 218.
2. Pappenheimer, J. R., and Kinter, W. B. Hematocrit ratio of blood within mammalian kidney and its significance for renal hemodynamics. Amer. J. Physiol. 1956, **185**, 377.
3. Lilienfield, L. S., Rose, J. C., and Porfido, F. A. Evidence for a red cell shunting mechanism in the kidney. Circulat. Res. 1957, **5**, 64.
4. Ochwadt, B. Durchflusszeiten von Plasma und Erythrocyten, intrarenaler Hämatokrit und Widerstandsregulation der isolierten Niere. Pflüg. Arch. ges. Physiol. 1957, **265**, 112.
5. Lassen, N. A., Longley, J. B., and Lilienfield, L. S. Concentration of albumin in the renal papilla. Clin. Res. 1958, **6**, 154.
6. Lilienfield, L. S., Lassen, N. A., and Rose, J. C. The diverse distribution of red cells and albumin within anatomic regions of the kidney. Proc. Southern Society Clin. Research, Twelfth Annual Meeting, New Orleans, La., January 25, 1958, p. 32.
7. Pease, D. C. Electron microscopy of the vascular bed of the kidney cortex. Anat. Rec. 1955, **121**, 701.

SERIAL CHANGES IN TISSUE CARBON DIOXIDE CONTENT DURING ACUTE RESPIRATORY ACIDOSIS [1, 2]

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The retention of CO_2 in man and animals in chronic respiratory disease and during exposure to atmospheres with high partial pressures of CO_2 was first shown a number of years ago (1–4). Its converse, the reduction of CO_2 stores during hyperventilation, was demonstrated at the same time. However, considerable doubt has existed as to the site in the body occupied by this store of CO_2, how rapidly it equilibrates with blood and alveolar CO_2, and what effects its accumulation may have on acid-base equilibria in the extracellular and intracellular compartments.

Brocklehurst and Henderson (1) found small changes in tissue CO_2 stores in man in experiments lasting only two to three minutes, while Shaw (2), using cats and a longer equilibration time, postulated that as much as 80 per cent of the CO_2 taken up was "buffered" by the tissues. Ferguson, Irving, and Plewes (3, 4) attempted to localize the area of storage, but were unable to account for all the CO_2 taken up in cats although they found increases in blood, muscle, brain and kidney CO_2 content. They pointed out that bone contained 40 to 50 per cent of the total body CO_2 and were able to lower bone CO_2 3 per cent by hyperventilation, but they failed to raise the bone CO_2 content by increasing the CO_2 tension in the inspired atmosphere for several hours. More recently, however, Freeman and Fenn found the bone CO_2 of rats increased after exposure to 10 per cent CO_2 in air for periods of 6 to 28 days (5). They also found considerable increases in muscle CO_2. On the other hand, in brief (six hours) hyperventilation experiments with low oxygen tensions they were not able to mobilize bone CO_2 to any appreciable extent. Thus the site of storage of retained CO_2, especially in relatively short experiments, has remained in considerable doubt. Furthermore, none of these authors commented to any extent on the changes in acid-base balance which one might expect from such changes in the alveolar CO_2 tension and the total CO_2 content of the body.

The features of the clinical pattern seen in man with chronic retention of CO_2 due to respiratory disease are well known (6), and the chemical findings of this syndrome have been described (7). In this regard, Schaefer (8), studying the possible toxic effects of this gas, has shown that during prolonged exposure of man to 3 to 5 per cent CO_2 an adaptive period of respiratory acidosis is followed by a phase of acclimatization during which plasma pH returns to normal and respiratory stimulation disappears. An increase in the body stores of CO_2 during the adaptation to these high CO_2 atmospheres was indicated by the large excretion of CO_2 by lungs and kidneys during reacclimatization to normal air.

Moreover, a recent series of studies by Schaefer and Nichols (9) of prolonged breath-holding in man during dives to depths of 100 feet (equivalent to an increase in pressure of three atmospheres) have demonstrated that the tissue stores of CO_2 can be greatly increased in a very short period of time (two minutes) without any change occurring in the pH or total CO_2 of the plasma. Unfortunately, no data indicating the tissues in which this CO_2 was stored could be obtained from these experiments.

The effect of increasing tissue cell CO_2 on the acid-base balance of the intracellular space has not been studied extensively. However, Wallace and Hastings (10) and Wallace and Lowry (11) found that the intracellular bicarbonate varied directly with the CO_2 tension and not with the bicarbonate ion concentration of the surrounding

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fluids. Thus intracellular pH varied inversely with extracellular CO_2 tension.

The doubts thus raised by others concerning the relation between the duration of exposure and location of CO_2 storage and the effects of CO_2 storage on the organism led us to re-examine the CO_2 contents of several tissues in rats at varying times following the initiation of exposure to an atmosphere containing 24 per cent CO_2 in air. The aims of the experiment were to discover where in the body CO_2 was stored after various times of exposure and what effect if any this storage might have upon the acid-base balance of the various tissues studied. The data presented below indicate that in the intact rat, exposed for periods up to 48 hours to this atmosphere, the cells of the soft tissues and the extracellular phase were the site of storage of the CO_2 retained and were in equilibrium with the alveolar CO_2 tension. In contrast, bone mineral CO_2 appeared to be in equilibrium with the carbonate ion [5] concentration in extracellular water rather than with alveolar or extracellular pCO_2, and therefore did not serve as a repository for the CO_2 accumulated by these animals.

METHODS

Ninety-three male albino rats weighing 290 to 400 Gm. were used. All rats were at least 120 days old, at which time bone CO_2 has stabilized (12). The majority were of the Wistar strain while 10 were of the Sprague-Dawley strain. No difference between the two strains was found except after 15 hours of exposure to CO_2 where the Sprague-Dawley rats seemed to have a higher mortality rate. Eighty-three of the rats were exposed in individual chambers for periods ranging from one-half to 48 hours to an atmosphere containing 24 per cent CO_2 in air. The CO_2 content of the atmosphere was checked using the manometric method of Van Slyke (13). At the end of the period of exposure the animals were lightly anesthetized with pentobarbital intraperitoneally and exsanguinated through the abdominal aorta, using oiled syringes and 0.1 ml. of sodium heparin to prevent clotting. Samples of muscle, brain and bone were obtained as quickly as possible from the carcass. Exposure to the experimental atmosphere was continued through the period of anesthesia and sacrifice by means of a mask. Ten control rats were sacrificed in the same manner. All animals had access to water and food *ad libitum*. The rats exposed to high tensions of CO_2 ate and drank very little compared to the controls. While this probably had no effect on the composition of the tissues of rats exposed for short periods, it might have produced some differences in those exposed for longer intervals.

The following measurements were made: Whole blood hematocrit was estimated in Wintrobe tubes centrifuged for 30 minutes at 4,000 rpm in an International centrifuge with a no. 240 head. Whole blood CO_2 was measured by the manometric method of Van Slyke and Neill (14). pH was determined at 38° C. anaerobically using a Beckman glass electrode type 290–31 and a Model G pH meter. Plasma water was measured by a method previously described (17) and plasma chloride in duplicate by the method of Wilson and Ball (15).

Muscle and brain [6] samples were divided into two parts immediately following excision. One was instantly placed in a tube containing alkaline FeF_3 as a metabolic inhibitor and the total CO_2 was determined by the method of Danielson and Hastings (16). Muscle, water, fat, and chloride were measured by methods previously described (17). Water and chloride were determined in brain by the same procedures, but no extraction of fat was carried out since such an extraction has been shown to remove electrolyte from this tissue (18).

Bone water and chloride were measured by methods already reported (19). Bone CO_2 was determined on the dry powdered bone by a modification of the method of Danielson and Hastings (16). An ash weight was also determined for each bone sample following 48 hours in an electric muffle oven at 550° C.

Calculations. Plasma total CO_2 was determined from the whole blood CO_2, the hematocrit, and pH using Van Slyke and Sendroy's nomogram (20). pCO_2 and plasma bicarbonate were calculated from the formulae of Singer and Hastings using a CO_2 solubility factor for plasma of 0.0302 and a pK of 6.10. All calculations were made on the basis of a temperature of 38° C. Concentrations in plasma water were calculated by the formula previously reported (21).

The total CO_2 of fat-free wet muscle was calculated by subtracting the CO_2 dissolved in the fat of the muscle sample from the total contained in the sample analyzed. It was assumed that the pCO_2 of adipose tissue was the same as plasma. A CO_2 solubility factor for the depot fat of 0.0577 was used (22).

The volume of the extracellular fluid of the tissue samples was assumed to be equal to the chloride space and calculated by the formulae of Hastings and Eichelberger (23). A Donnan distribution factor of 0.98 was used for the calculation of extracellular chloride concentration (24). The volume of intracellular water was obtained by subtracting the extracellular fluid water from the total tissue water. The concentration of HCO_3^- in extracellular water was calculated from the values for plasma water divided by a Donnan factor of 0.99 (24), and the carbonic acid of this phase by assuming that the solubility factor for CO_2 and the pCO_2 in ex-

[5] $[CO_3^{=}] = K_2 \frac{[HCO_3^-]}{[H^+]}$ where K_2 is the second dissociation constant of carbonic acid.

[6] The brain was removed *in toto* and split longitudinally into two equal parts.

tracellular water were the same as for plasma. Extracellular carbonate ($CO_3^=$) concentration was calculated from the extracellular water (HCO_3^-) and the pH of plasma by the Henderson-Hasselbalch equation using a pK of 9.76.

In calculating the intracellular concentrations of H_2CO_3 and bicarbonate in muscle and brain, the total CO_2 contained in the extracellular water was subtracted from the total for the tissue sample. This total intracellular CO_2 was then apportioned between H_2CO_3 and HCO_3^- in the following manner. It was assumed that the pCO_2 in intracellular fluid was the same as in all other body phases. This was multiplied by a CO_2 solubility factor for muscle and brain water of 0.0276 [7] to obtain the concentration of H_2CO_3. The H_2CO_3 was subtracted from the total CO_2 to obtain the HCO_3^-. Finally, intracellular pH was estimated from the Henderson-Hasselbalch equation using a pK of 6.10 and assuming no CO_2 was bound within the cell.

Bone mineral $CO_3^=$ was determined by subtracting the small amount of CO_2 contained in the extracellular water of bone from the total CO_2 and expressing the remainder in terms of bone ash weight.

RESULTS

The responses of the individual animals to the atmosphere containing 24 per cent CO_2 varied considerably. Some became moribund in a short time, while others after a few hours were able to move about and take food and water. All, however, showed marked hyperpnea and moderate to marked lethargy intermixed with periods of extreme restlessness. This great variability in response was reflected in a large variability from animal to animal in the analytic values for the tissues studied. No significant correlation between the variations of response and the variation in tissue analysis could be found. However, despite the considerable differences which occurred from animal to animal, certain trends were uniformly apparent.

Table I presents the means and standard deviations of the means of the analytic values found in plasma, muscle, brain and bone after various periods of exposure to CO_2. The figures in parentheses following each mean value indicate the number of individual animals represented by each mean. It can be seen from this table that exposure to 24 per cent CO_2 in air produced a prompt profound respiratory acidosis with a marked rise in arterial pCO_2 and total plasma CO_2. Plasma chloride and water decreased to a smaller extent. These changes were accompanied by a rise in the CO_2 content of both muscle and brain, but no apparent change in bone CO_2. The other values for tissues showed only minor changes.

Figure 1 presents the plasma values in graphic form and illustrates the degree of acidosis of the extracellular fluids found in these animals after exposure to CO_2 for various times. The time scale for the first hour of exposure has been expanded for the sake of clarity in this and subsequent figures. The vertical bars about each point represent one standard deviation on each side of the mean. The pCO_2 rose in the first 30 minutes to 180 mm. of mercury. There appeared to be a slight further rise reaching a peak at 15 to 24 hours and a slight decline at 48 hours, but these values are not significantly different from the half-hour value. It is of interest to note that the pCO_2 for the arterial plasma (presumably equal to alveolar pCO_2) appeared higher than that of the inspired air (180 mm.) from the fifth hour onward. The plasma bicarbonate concentration rose rapidly in the early hours of exposure but not as rapidly as the pCO_2. However, as exposure continued, plasma bicarbonate continued to rise at a slow but steady rate although the pCO_2 remained relatively stable. The early lag in bicarbonate accretion resulted in a very low pH after relatively short exposures, while the later rise in bicarbonate without an equivalent increase in pCO_2 resulted in some return of pH toward normal. These changes in plasma values correlate well with the chloride concentrations and are in keeping with the findings of other workers (25, 26). They sug-

[7] This figure represents the average of four determinations carried out on fat-free homogenates of muscle and brain at pH 4.01 and 38° C. by a method similar to that previously described from this laboratory (22). This value is somewhat lower than that estimated by Danielson, Chu, and Hastings (35) and used in their calculations for the pK_1 of carbonic acid in muscle. It was used in these calculations since it represented a direct measurement on the tissues concerned rather than an estimate based on the general effect of proteins on the solubility of CO_2 in aqueous solutions. Although lower values for H_2CO_3 in all tissues and hence higher pH's were obtained thus than would otherwise have been the case and therefore the absolute values for pH are open to some question, the differences in pH, compared to the controls, which occurred during the experimental periods are not affected.

TABLE I

Tissue composition of rats exposed to 24 per cent CO_2 in air—Basic data (mean values and standard deviations)

Exposure	Plasma					Muscle				Brain			Bone			
	pH	pCO_2	Total CO_2	H_2O	Cl^-	H_2O	Fat	Cl^-	Total CO_2	H_2O	Cl^-	Total CO_2	H_2O	Cl^-	Total CO_2	Ash weight
hours		*mm. Hg*	*mM/L.*	*Gm./L.*	*mM/L.*	*Gm./ Kg. FFT*	*Gm./ Kg. tissue*	*mM/ Kg. FFT*	*mM/Kg. fatty tissue*	*Gm./ Kg. tissue*	*mM/ Kg. tissue*	*mM/ Kg. tissue*	*Gm./ Kg.*	*mM/ 100 Gm. dry sol.*	*mM/ 100 Gm. dry sol.*	*% of total dry sol.*
0	7.47 (10)*	36.3 (10)	26.7 (10)	936 (5)	100.2 (10)	766 (5)	10.1 (5)	10.7 (5)	11.7 (5)	774 (5)	31.3 (5)	12.7 (5)	237 (10)	2.2 (10)	87.0 (10)	72.2 (10)
	S.D. 0.06†	S.D. 5.5	S.D. 2.8	S.D. 3.1	S.D. 2.7	S.D. 4.0	S.D. 2.5	S.D. 1.2	S.D. 1.0	S.D. 5.4	S.D. 5.9	S.D. 2.4	S.D. 13.1	S.D. 0.01	S.D. 5.0	S.D. 1.0
1/2	6.92 (5)	180 (5)	40.4 (5)	936 (2)	97.4 (5)	760 (5)	10.4 (5)	15.5 (5)	17.3 (5)	773 (5)	33.2 (5)	23.3 (5)	252 (5)	2.1 (5)	80.4 (4)	72.6 (3)
	S.D. 0.03	S.D. 13.0	S.D. 1.6	S.D. 1.4	S.D. 4.4	S.D. 3.4	S.D. 5.5	S.D. 1.6	S.D. 1.9	S.D. 5.7	S.D. 1.9	S.D. 3.0	S.D. 29.3	S.D. 0.3	S.D. 5.6	S.D. 1.3
1	7.02 (10)	162 (9)	43.8 (9)	928 (5)	94.6 (10)	766 (5)	14.7 (5)	11.3 (5)	18.9 (4)	780 (5)	32.3 (5)	25.9 (4)	235 (8)	2.1 (5)	84.0 (5)	72.1 (4)
	S.D. 0.02	S.D. 11.0	S.D. 2.3	S.D. 2.2	S.D. 2.1	S.D. 6.0	S.D. 6.0	S.D. 1.5	S.D. 3.6	S.D. 7.9	S.D. 1.9	S.D. 3.3	S.D. 27.1	S.D. 0.6	S.D. 2.2	S.D. 5.3
3	7.00 (9)	182 (9)	47.4 (9)	928 (3)	92.4 (9)	768 (6)	17.5 (6)	11.0 (6)	24.3 (6)	788 (5)	29.8 (5)	31.9 (5)	256 (9)	2.2 (9)	83.0 (8)	74.2 (9)
	S.D. 0.06	S.D. 17.0	S.D. 1.3	S.D. 3.1	S.D. 3.4	S.D. 4.6	S.D. 28.9	S.D. 1.7	S.D. 4.8	S.D. 13.1	S.D. 1.9	S.D. 2.2	S.D. 22.4	S.D. 0.16	S.D. 3.4	S.D. 2.6
5	6.96 (7)	205 (7)	51.0 (8)	926 (5)	91.2 (8)	764 (7)	14.4 (7)	11.3 (7)	28.1 (7)	776 (5)	29.9 (5)	32.5 (4)	250 (7)	2.0 (7)	80.3 (7)	72.8 (7)
	S.D. 0.05	S.D. 21.0	S.D. 1.5	S.D. 3.5	S.D. 4.5	S.D. 6.6	S.D. 9.2	S.D. 1.8	S.D. 5.4	S.D. 3.9	S.D. 1.2	S.D. 0.6	S.D. 17.3	S.D. 0.23	S.D. 3.5	S.D. 1.2
7	6.99 (7)	205 (7)	52.6 (7)	924 (6)	89.0 (7)	765 (6)	15.4 (6)	11.9 (6)	26.7 (6)	777 (5)	27.7 (5)	32.1 (5)	235 (7)	1.9 (7)	85.5 (5)	73.5 (7)
	S.D. 0.04	S.D. 18.0	S.D. 1.7	S.D. 3.4	S.D. 4.1	S.D. 9.2	S.D. 6.9	S.D. 1.5	S.D. 3.2	S.D. 6.9	S.D. 1.6	S.D. 4.2	S.D. 39.6	S.D. 0.37	S.D. 6.2	S.D. 1.2
15	6.96 (6)	216 (6)	51.8 (6)	920 (1)	94.2 (5)	764 (4)	15.8 (4)	10.3 (4)	27.8 (4)	777 (4)	28.1 (4)	36.1 (4)	249 (9)	2.1 (10)	84.1 (10)	73.2 (10)
	S.D. 0.09	S.D. 22.0	S.D. 3.8		S.D. 4.3	S.D. 13.4	S.D. 6.1	S.D. 1.3	S.D. 3.4	S.D. 9.3	S.D. 0.3	S.D. 4.4	S.D. 28.2	S.D. 0.37	S.D. 4.2	S.D. 1.7
24	7.01 (6)	214 (6)	57.7 (6)	923 (4)	90.1 (6)	763 (6)	23.5 (6)	12.4 (6)	24.5 (6)	783 (4)	27.8 (4)	36.2 (4)	261 (5)	2.1 (6)	82.1 (6)	72.2 (6)
	S.D. 0.02	S.D. 14.0	S.D. 3.3	S.D. 2.5	S.D. 2.8	S.D. 7.3	S.D. 11.6	S.D. 2.1	S.D. 2.9	S.D. 8.2	S.D. 1.2	S.D. 4.6	S.D. 42.4	S.D. 0.39	S.D. 6.9	S.D. 3.8
48	7.10 (4)	196 (4)	63.9 (4)	922 (3)	82.4 (4)	752 (4)	7.8 (4)	11.3 (4)	27.7 (4)	774 (4)	26.9 (4)	37.0 (4)	222 (4)	1.7 (4)	82.8 (4)	72.6 (4)
	S.D. 0.01	S.D. 8.0	S.D. 1.8	S.D. 7.2	S.D. 1.7	S.D. 3.5	S.D. 2.5	S.D. 3.8	S.D. 3.2	S.D. 8.3	S.D. 1.7	S.D. 2.2	S.D. 35.5	S.D. 0.27	S.D. 5.4	S.D. 3.8

* Number of determinations.
† Standard Deviation.

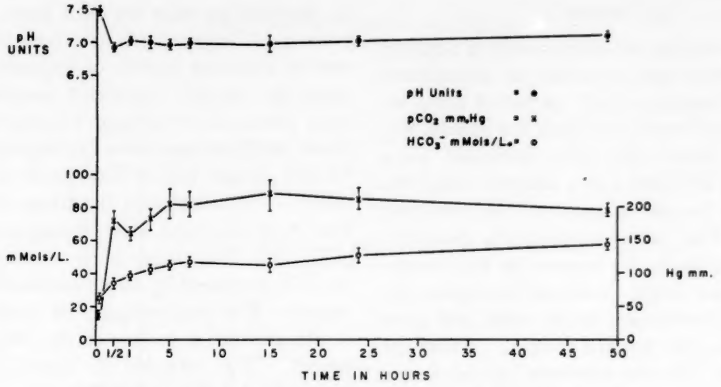

FIG. 1. PLASMA CHANGES DURING ACUTE RESPIRATORY ACIDOSIS

gest that our animals, at least by 24 to 48 hours, had passed beyond the phase of acute acidosis and were entering a phase of adaptation to the abnormal atmosphere.

The mean values for total CO_2 found in the various tissues are plotted in Figure 2, with the total plasma CO_2 included for comparison. The values are expressed in terms of 1 Kg. of wet tissue for plasma, brain and for muscle after correction for fat. The accumulation of CO_2 in brain and muscle appeared to proceed in a similar fashion, except that the total CO_2 content of brain rose more rapidly and reached higher values than was the case in muscle. This difference between the total CO_2 of brain and muscle was statistically significant ($p < 0.05$) in six of the eight periods of exposure studied.

With exposures up to five hours the curves of muscle and brain CO_2 paralleled the plasma CO_2 almost exactly. However, beyond five hours of exposure the total CO_2 of these tissues remained stable while plasma CO_2 continued to rise slowly. In sharp contrast to the total CO_2 of plasma, muscle and brain, the total bone CO_2 showed remarkably little change. In fact, there was a decrease in the mean values of bone CO_2 of about 7 per cent while the muscle and brain CO_2 content rose 140 per cent.

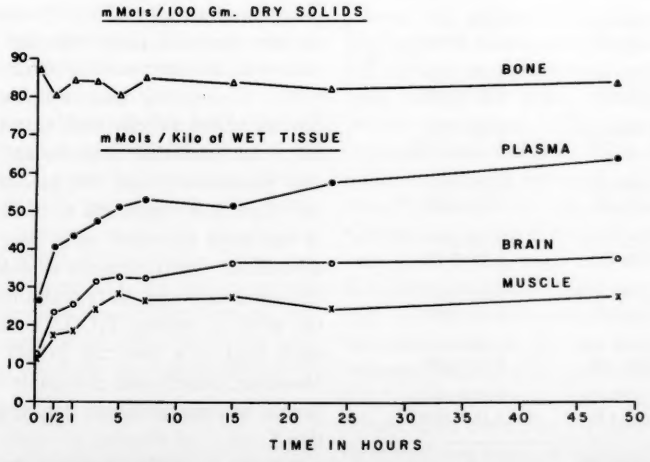

FIG. 2. DISTRIBUTION OF TOTAL CO_2 DURING ACUTE RESPIRATORY ACIDOSIS

DISCUSSION

These data confirm the observations of Schaefer in man (8) that upon exposure to atmospheres rich in CO_2 there is an early period of acute respiratory acidosis with hyperpnea, low plasma pH, and lowered urine pH with decreased urine HCO_3^-. This is followed by a phase in which the organism has become adapted to the abnormal atmosphere. This adaptive period was characterized in his studies by an increase in CO_2 excretion through the lungs, decreased hyperpnea, the appearance of bicarbonate in the urine, and a return of plasma pH toward normal. From the work of Shaw (2) the inference can be drawn that this adaptive period does not begin until after tissue stores of CO_2 have reached an equilibrium with the increased pCO_2 of the alveolar air. Our findings for muscle and brain CO_2 conform with this hypothesis since the CO_2 content of these two tissues had reached a plateau at five hours, and the adaptive change in plasma pH did not appear until much later.

A further confirmation might be drawn from the establishment of a plasma pCO_2 above that of the inspired air after the fifth hour. During the process of "saturation" described by Shaw and seen in the short periods of exposure of our animals, the normal downward gradient of pCO_2 from tissue cell to ambient air must be reversed. Under such circumstances the respiratory quotient (R.Q.) would fall to low levels as Shaw has shown. However, once the tissue stores of CO_2 have been saturated, some downward gradient of pCO_2 from tissue to air must be re-established for the CO_2 produced by tissue metabolism to be excreted. The reappearance of such a gradient would produce a return of the R.Q. to normal ranges. This occurred in Shaw's experiments. It should also be evidenced by the appearance of a higher pCO_2 in the arterial blood than is found in the inspired air as was demonstrated here.

TABLE II

Comparison of changes in extracellular water and bone mineral carbonate concentration

Exposure	$CO_3^=$ Bone	$CO_3^=$ E.C.W.
hrs.	*mM/Kg. mineral*	*mM/Kg. H_2O*
0	1,185 (9)*	0.151 (10)
	S.D. 60†	S.D. 0.036
1/2	1,099 (4)	0.053 (4)
	S.D. 66	S.D. 0.002
1	1,156 (5)	0.074 (8)
	S.D. 60	S.D. 0.006
3	1,109 (8)	0.082 (9)
	S. D. 38	S. D. 0.013
5	1,090 (7)	0.079 (8)
	S.D. 38	S.D. 0.011
7	1,151 (5)	0.086 (7)
	S.D. 81	S.D. 0.010
15	1,138 (10)	0.080 (6)
	S.D. 55	S.D. 0.022
24	1,129 (6)	0.099 (6)
	S.D. 140	S.D. 0.007
48	1,127 (4)	0.139 (4)
	S.D. 68	S.D. 0.002

* Number of determinations.
† S.D., standard deviation.

The bone CO_2 values, however, do not fit into this concept that all tissue CO_2 stores reach a rapid equilibrium with alveolar pCO_2. The apparent discrepancy between the bone CO_2 values found after short exposure to CO_2 (3, 4) compared to prolonged exposure (5) suggested to us that the bone CO_2 content was not in equilibrium with alveolar pCO_2 in our experiments but rather was related to some other parameter which changed more slowly during the various stages of adaptation to high CO_2 atmospheres.

Underwood, Toribara, and Neuman, studying the CO_2 of bone and synthetic apatites with infrared spectroscopy, have shown that bone mineral CO_2 is entirely present as $CO_3^=$ ion (27). They have also shown that the $CO_3^=$ content of synthetic apatites varies directly with the HCO_3^- concentration in the surrounding fluids at constant pH (28). Evidence is also available that bone CO_2 content varies directly with extracellular pH (29, 30). In metabolic acidosis and alkalosis these two parameters vary in parallel fashion, and, therefore, it is impossible to distinguish the effect of one from the other upon bone carbonate concentration. In respiratory acidosis and alkalosis, however, they vary in opposite directions. Thus, the effect of raising HCO_3^- concentration might mask that of a decrease in pH and vice-versa. However, bicarbonate is capable of dissociating a proton to form carbonate ion according to Equation I.

$$\text{I} \qquad HCO_3^- \rightleftarrows H^+ + CO_3^=$$

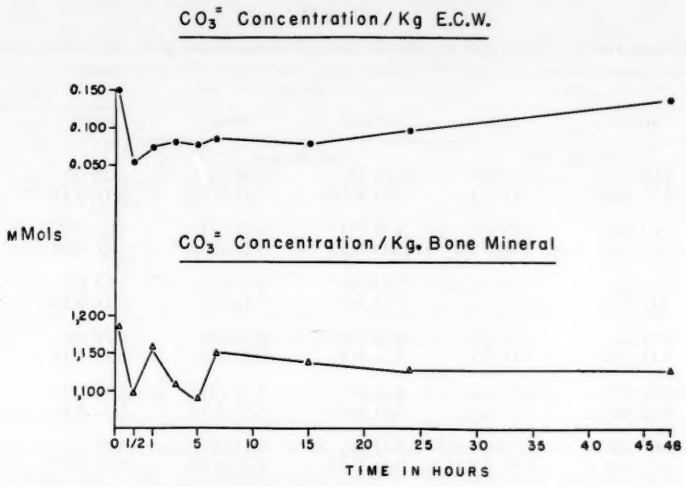

FIG. 3. DISTRIBUTION OF CARBONATE (mM PER KG. EXTRACELLULAR WATER) DURING ACUTE RESPIRATORY ACIDOSIS

The pK of this reaction is 9.76. Thus, by calculating the $CO_3^=$ ion concentration in extracellular fluid, it is possible to express the effects of variations in *both HCO_3^- and pH in a single term* [8] and assess their *combined actions* upon bone CO_2 content. Such an expression has the additional appeal of suggesting that bone crystal $CO_3^=$ is in equilibrium with the $CO_3^=$ ion concentration in the surrounding fluids, although it is obviously impossible to say whether the proton is dissociated from the bicarbonate in the crystal surface or in the surrounding fluid. In order to examine this proposition the concentrations of carbonate in extracellular water and in bone mineral for each period of exposure were calculated.

The reasons for calculating the bone mineral carbonate concentration were as follows: Of the total amount of CO_2 found in bone about 99 per cent is associated with the mineral salts, while only 1 per cent is present as H_2CO_3 and HCO_3^- in the extracellular fluid of this tissue. Thus, correction of the total bone CO_2 for that contained in the extracellular fluid even if that amount was doubled made no significant difference in the final value. However, acidosis has been shown to cause the release of mineral salts from bone (29, 30) and, therefore, values for bone CO_2, expressed in terms of the dry solids of bone, might be misleading due to changes in the ratio between organic and mineral solids in certain of the animals.

The results of these calculations are shown in Table II and Figure 3. It can be seen that, despite the marked increase in total CO_2 in the plasma (Figure 2), the concentration of $CO_3^=$ in the extracellular water fell 65 per cent in the first 30 minutes of exposure due to the sharp fall in plasma pH (Figure 1). It remained at a low level up to 15 hours, then rose slowly as the plasma pH slowly returned toward normal and the animals entered the phase of adaptation to the abnormal atmosphere. The values of bone mineral carbonate also decrease. This decrease is more rapid in the early hours of exposure, leveling off after 24 hours. No rise is apparent at 48 hours. Unfortunately, the groups of animals exposed for each period were small and the individual variations between animals were very great (see Table II). For these reasons the shape of the bone mineral $CO_3^=$ curve is in some doubt, and no precise estimate can be made of the ratio of extracellular $CO_3^=$ concentration to bone mineral $CO_3^=$ concentration from these data. However, the values for three and five hours of exposure are significantly lower than the controls ($p < 0.02$ and < 0.01, re-

[8] It is apparent that small changes in pH will have a relatively greater effect on extracellular water $CO_3^=$ concentration than similar changes in HCO_3^- concentration since the former is an exponential term.

TABLE III

Changes in the carbonic acid, bicarbonate, and pH of muscle and brain intracellular water

Exposure	HCO_3^-		H_2CO_3		pH Units	
	Muscle	Brain	Muscle	Brain	Muscle	Brain
hrs.	*mM/Kg. H_2O*		*mM/Kg. H_2O*			
0	12.5 (5)* S.D. 2.0†	10.3 (5) S.D. 2.1	1.01 (5) S.D. 0.56	1.00 (5) S.D. 0.08	7.20 (5) S.D. 0.11	7.11 (5) S.D. 0.06
1/2	11.1 (5) S.D. 1.3	18.3 (4) S.D. 3.8	4.97 (5) S.D. 0.36	4.86 (4) S.D. 0.33	6.46 (5) S.D. 0.08	6.69 (4) S.D. 0.11
1	16.4 (4) S.D. 5.3	19.7 (4) S.D. 8.8	4.35 (4) S.D. 0.42	4.55 (4) S.D. 0.23	6.65 (4) S.D. 0.19	6.70 (4) S.D. 0.22
3	23.0 (6) S.D. 7.6	29.7 (5) S.D. 5.1	4.84 (6) S.D. 0.19	5.10 (5) S.D. 0.53	6.77 (6) S.D. 0.14	6.86 (5) S.D. 0.09
5	26.1 (7) S.D. 8.8	27.1 (4) S.D. 1.8	5.66 (7) S.D. 0.57	5.92 (4) S.D. 0.72	6.75 (7) S.D. 0.14	6.77 (4) S.D. 0.05
7	24.0 (6) S.D. 5.6	26.9 (5) S.D. 8.7	5.75 (6) S.D. 0.50	5.67 (5) S.D. 0.65	6.72 (6) S.D. 0.12	6.76 (5) S. D. 0.16
15	27.2 (4) S.D. 4.9	34.9 (4) S.D. 8.0	5.61 (4) S.D. 0.24	7.10 (4) S.D. 0.7	6.78 (4) S.D. 0.07	6.78 (4) S.D. 0.12
24	20.0 (6) S.D. 4.9	30.6 (4) S.D. 7.5	5.92 (6) S.D. 0.39	6.05 (4) S.D. 0.35	6.62 (6) S.D. 0.11	6.80 (4) S.D. 0.09
48	24.5 (4) S.D. 6.4	28.9 (4) S.D. 2.9	5.42 (4) S.D. 0.31	5.42 (4) S.D. 0.31	6.74 (4) S.D. 0.14	6.83 (4) S.D. 0.04

* Number of determinations.
† S.D., standard deviation.

spectively). Furthermore, when the mean value for all the experimental animals is compared to the mean of the controls, it appears to be significantly lower ($p = 0.02$). These data suggest that the bone mineral carbonate *in vivo* is in equilibrium with the carbonate ion concentration of the extracellular fluids, as postulated above, rather than with the alveolar CO_2 tension or the total CO_2 of the plasma.

Such an hypothesis offers an explanation for the divergence between the total bone CO_2 levels seen in short-term experiments such as are presented here and long-term exposures to atmospheres high in CO_2. Thus, after the 6 to 28 days of exposure to 10 per cent CO_2 in air required by Freeman and Fenn's (5) animals to show rises in bone CO_2, adaptation should have been quite complete with high plasma bicarbonate concentrations and pH values approaching the normal range. Under such circumstances extracellular $CO_3^=$ concentration would be elevated and thus bone $CO_3^=$ should be similarly increased.

The results of these authors' experiments with rats in which hyperventilation was induced by low O_2 tensions also fit this postulation. Following hyperventilation of only six hours, total bone CO_2 did not change, while in long experiments (11 to 31 days) bone CO_2 decreased appreciably. Although no pH values for plasma are reported, one would expect alkalosis with a high pH as well as a lower extracellular HCO_3^- concentration in the six hour animals and thus little change in extracellular $CO_3^=$ concentration. In the latter experiments adaptation may well have occurred so that the pH of the extracellular fluid was approximately normal. If this assumption is correct, the observed reduction in extracellular bicarbonate was accompanied by a reduction of $CO_3^=$ concentration and therefore a decrease in bone $CO_3^=$.

The apparent failure of bone $CO_3^=$ concentration to rise with the plasma $CO_3^=$ concentration in the last two experimental periods cannot be explained from the data presented here. One can only speculate whether this is merely an artifact due to wide variations in values and small groups of animals or whether there is some delay of

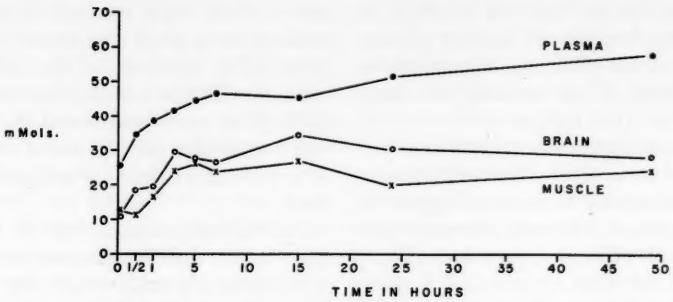

FIG. 4. DISTRIBUTION OF BICARBONATE (mM PER KG. EXTRACELLULAR WATER) DURING ACUTE RESPIRATORY ACIDOSIS

equilibration due to slow diffusion in the relatively poorly vascularized bone. It is also possible that some local change may have occurred in the bone itself which influenced the $CO_3^=$ ion distribution between mineral and extracellular fluid. Further work will be needed to answer these questions.

The increases in CO_2 content of muscle and brain presented in Table I and Figures 1 and 2 are considerable. Although the increases in extracellular fluid CO_2 content of these animals was large, the total increases in CO_2 in these tissue samples could not be accounted for on the basis of changes in the extracellular concentration alone unless the extracellular volumes of the samples obtained were severalfold those usually found (17). Therefore, it was apparent that the intracellular CO_2 content must have changed. In order to evaluate these changes and examine what effect storage of CO_2 might have had on the acid-base balance of these two tissues, the concentrations of bicarbonate, carbonic acid, and hydrogen ion in the intracellular water of both muscle and brain were calculated for each animal. The averages of the results obtained are presented in Table III and have been plotted graphically in Figures 4 and 5 for comparison with the plasma.

It can be seen in Figure 4 that the bicarbonate ion concentration in both brain cells and muscle fibers increased rapidly, reaching a plateau after only three hours of exposure. The accumulation of bicarbonate in the cells of these tissues was considerably more rapid than the accumulation in the plasma since the latter did not reach the slower phase of its accumulation until after seven hours. However, once the cells had accumulated bicarbonate, their stores of this ion remained relatively fixed and if anything tended to decrease slightly with prolonged exposure. Bicarbonate accumulated in both these cell types in a very similar fashion except for the first 30 minutes of exposure. While brain cell bicarbonate almost doubled during this period, muscle fiber bicarbonate did not

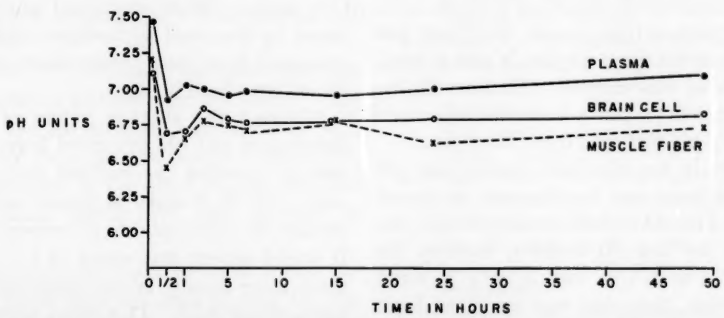

FIG. 5. COMPARISON OF CALCULATED pH OF MUSCLE FIBERS AND BRAIN CELLS WITH PLASMA pH

change, the sharp rise in HCO_3^- in the latter tissue cell occurring between 30 and 60 minutes. The probability of this difference in response between the two types of cell occurring by chance was only 1 per cent ($p = 0.01$).

Although muscle intracellular bicarbonate concentrations rose more slowly than did those of brain and did not appear to reach as high levels, there was no significant difference between the two tissues except at 30 minutes of exposure. This is in contrast to the values for total tissue CO_2 shown in Figure 2. The difference in statistical significance between the two forms of expression lies in the correction made for extracellular fluid CO_2. The extracellular volumes found in brain were larger than in muscle and thus the fraction of the total CO_2 assigned to the extracellular fluid in this tissue was not only larger but tended to increase more rapidly than in muscle due to the rising extracellular fluid bicarbonate concentration in the longer exposure periods.

These findings are compatible with the concept that the cell membranes of these two tissues are relatively impermeable to bicarbonate ion but freely permeable to CO_2 since the ratio of extracellular to intracellular bicarbonate varies both with the tissue and with time. Assuming as we have that the pCO_2 is the same at all times in all body phases and having found that the solubility coefficient for CO_2 in brain and muscle cell water is the same, then the rate of accumulation of bicarbonate in these cells will depend on the rate at which CO_2 becomes hydrated to form H_2CO_3, which in turn dissociates to give bicarbonate ion. The enzyme carbonic anhydrase is known to accelerate the hydration of CO_2. Since this enzyme is present in appreciable quantities in brain (31) but is virtually absent from muscle, the slower rise of bicarbonate in this tissue may be, in part at least, due to a lack of this enzyme. Further experiments are needed to prove such an hypothesis which can only be suggested from these data.

The changes in the calculated intracellular pH of muscle and brain may be compared to plasma in Figure 5. The pH of both these cells falls precipitously in the first 30 minutes, as does the plasma pH. The fall in muscle cells is much greater, however, than that seen in either brain or plasma. After one hour the muscle cell pH had risen again to the same level as that of brain cells. From then on both remained relatively constant at a level considerably below the controls. The constancy of the pH of these cells even after 48 hours of exposure may be contrasted to the slow rise in plasma pH which seems to occur over similar periods during which the animals are showing evidence of adaptation to the CO_2 load.

Calculations such as these of the intracellular bicarbonate, carbonic acid, and hydrogen ion concentrations are subject not only to the possible errors involved in the use of a chloride space (17) [9] but also those inherent in the assumption that the pCO_2 of all body phases is the same as that in the alveolus at all times. Although the rate of diffusion of CO_2 is not infinitely great, as implied by this assumption, it is known to be rapid and, therefore, whatever differences in CO_2 tension existed between the alveoli and the tissue cells at various periods of exposure were probably not large.

We have also assumed, as did Wallace, Hastings, and Lowry (10, 11), that no CO_2 was bound intracellularly. This view has been challenged by Conway and Fearon (33). However, at the level of pH ascribed to intracellular water by either Wallace and associates or Conway and Fearon, the amount of CO_2 bound as carbamate should be small (34). The binding of CO_2 in some other form is certainly suggested by Conway's experiments, but as yet no information is available concerning either the nature of such binding or the factors which might influence it. In this regard, the first pK for carbonic acid determined for muscle by Danielson, Chu, and Hastings (35) is identical to that of plasma in which little bound CO_2 exists. Were significant amounts of CO_2 bound by the usual intracellular contents, the pK calculated from their observations on muscle brei should have been higher.

Although the absolute values of intracellular bicarbonate and pH presented here are therefore open to question (as outlined above), they give some idea of changes in tissues which occurred during the development of respiratory acidosis. It would appear that stores of CO_2 accumulated much more rapidly in the tissue cells than in the extracellular fluid. This rapid accumulation was

[9] This is especially true in the case of brain (32).

accompanied by the same profound fall in pH and marked acidosis as was seen in the plasma. Thus, during the early hours of exposure to high CO_2 tensions a comparable degree of acidosis existed in plasma and tissues. However, in the latter hours of exposure the adaptative changes which were seen in the plasma did not appear to occur in the tissue cells. No further rise in bicarbonate took place and the pH remained fixed at a low level. The persistence of some degree of intracellular acidosis, despite the return of plasma pH to normal, in individuals adapted to atmospheres with increased CO_2 tensions, which is suggested by these findings, might be one cause of the impaired cerebral cortical functions described by Schaefer after prolonged exposure to 3 per cent CO_2 (8) and seen in chronic respiratory disease (6, 7), the changes in the response of the respiratory and vasomotor centers seen in such individuals, and the decrease in the general metabolic rate of man (8) which has been described under these conditions. The proof of this hypothesis must await further work in which measurements of function and the results of direct tissue analysis are compared in the same animal.

SUMMARY

1. Eighty-three male albino rats were exposed to 24 per cent CO_2 in air for periods ranging from one-half to 48 hours, and the pH and CO_2 content of their blood and tissues was compared with control rats.

2. A profound respiratory acidosis with high plasma CO_2 and a plasma pH of 6.92 appeared after one-half hour of exposure. This was followed after 7 to 15 hours by a further slow rise in plasma CO_2 and a rising pH which reached 7.10 after 48 hours.

3. The total CO_2 of muscle and brain rose rapidly but reached a plateau after five hours. The rate of rise and the absolute level of tissue CO_2 was higher in brain than in muscle. Bone CO_2 content in contrast to the other tissues remained fixed or declined slightly even after 48 hours of exposure.

4. It was concluded from these data that the soft tissues rather than the bone formed the site of storage of CO_2 under the conditions of these experiments.

5. These findings suggest that the bone CO_2 may be in equilibrium with the carbonate ion concentration ($CO_3^=$) of the extracellular fluid rather than with the alveolar CO_2 tension as is the case in the soft tissues.

6. Calculations of intracellular pH and bicarbonate concentration in brain and muscle indicated that a profound acidosis developed rapidly in both these tissues. However, in contrast to plasma no evidence of adaptation with return of pH toward normal was observed up to 48 hours of exposure. The possible implications of such a persistent intracellular acidosis in the syndrome of adaptation to CO_2 retention are discussed.

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REFERENCES

1. Brocklehurst, R. J., and Henderson, Y. The buffering of the tissues as indicated by the CO_2 capacity of the body. J. biol. Chem. 1927, **72**, 665.
2. Shaw, L. A. The comparative capacity of the blood and of the tissue to absorb carbonic acid. Amer. J. Physiol. 1926–27, **79**, 91.
3. Ferguson, J. K. W., Irving, L., and Plewes, F. B. The source of expired CO_2 in decapitated, eviscerated cats. J. Physiol. 1929–30, **68**, 265.
4. Irving, L., Ferguson, J. K. W., and Plewes, F. B. The source of CO_2 expired and the site of its retention. J. Physiol. 1930, **69**, 113.
5. Freeman, F. H., and Fenn, W. O. Changes in carbon dioxide stores of rats due to atmospheres low in oxygen or high in carbon dioxide. Amer. J. Physiol. 1953, **174**, 422.
6. Dickens, C. Posthumous Papers of the Pickwick Club. London, Chapman and Hall, 1836, chap. IV.
7. Burwell, C. S., Robin, E. D., Whaley, R. D., and Bickelmann, A. G. Extreme obesity associated with alveolar hypoventilation, a Pickwickian syndrome. Amer. J. Med. 1956, **21**, 811.
8. Schaefer, K. E. Studies of Carbon Dioxide Toxicity (1.) Chronic CO_2 Toxicity in Submarine Medicine. U. S. Naval Medical Research Laboratory Report No. 181, 1951, vol. 10, p. 156.
9. Schaefer, K. E., and Nichols, G., Jr. Unpublished observations.
10. Wallace, W. M., and Hastings, A. B. The distribution of the bicarbonate ion in mammalian muscle. J. biol. Chem. 1942, **144**, 637.

11. Wallace, W. M., and Lowry, O. H. An in vitro study of carbon dioxide equilibria in mammalian muscle. J. biol. Chem. 1942, **144**, 651.
12. Kramer, B., and Shear, M. J. Composition of bone. IV. Primary calcification. J. biol. Chem. 1928, **79**, 147.
13. Peters, J. P., and Van Slyke, D. D. Quantitative Clinical Chemistry, Vol. II, Methods. Baltimore, Williams and Wilkins, 1932.
14. Van Slyke, D. D., and Neill, J. M. The determination of gases in blood and other solutions by vacuum extraction and manometric measurement. J. biol. Chem. 1924, **61**, 523.
15. Wilson, D. G., and Ball, E. G. A study of the estimation of chloride in blood and serum. J. biol. Chem. 1928, **79**, 221.
16. Danielson, I. S., and Hastings, A. B. A method for determining tissue carbon dioxide. J. biol. Chem. 1939, **130**, 349.
17. Nichols, G., Jr., Nichols, N., Weil, W. B., and Wallace, W. M. The direct measurement of the extracellular phase of tissues. J. clin. Invest. 1953, **32**, 1299.
18. Eichelberger, L., and Richter, R. B. Water, nitrogen, and electrolyte concentration in brain. J. biol. Chem. 1944, **154**, 21.
19. Nichols, G., Jr., and Nichols, N. Changes in tissue composition during acute sodium depletion. Amer. J. Physiol. 1956, **186**, 383.
20. Van Slyke, D. D., and Sendroy, J., Jr. Studies of gas and electrolyte equilibria in blood. XV. Line charts for graphic calculations by the Henderson-Hasselbalch equation, and for calculating plasma carbon dioxide content from whole blood content. J. biol. Chem. 1928, **79**, 781.
21. Nichols, G., Jr., and Nichols, N. Electrolyte equilibria in erythrocytes during diabetic acidosis. J. clin. Invest. 1953, **32**, 113.
22. Nichols, G., Jr. The solubility of carbon dioxide in body fat. Science 1957, **126**, 1244.
23. Hastings, A. B., and Eichelberger, L. The exchange of salt and water between muscle and blood. I. The effect of an increase in total body water produced by the intravenous injection of isotonic salt solutions. J. biol. Chem. 1937, **117**, 73.
24. Manery, J. F. Water and electrolyte metabolism. Physiol. Rev. 1954, **34**, 334.
25. Cooke, R. E., Coughlin, F. R., Jr., and Segar, W. E. Muscle composition in respiratory acidosis. J. clin. Invest. 1952, **31**, 1006.
26. Miller, A. T., Jr. Acclimatization to carbon dioxide: A study of chemical and cellular changes in the blood. Amer. J. Physiol. 1940, **129**, 524.
27. Underwood, A. L., Toribara, T. Y., and Neuman, W. F. An infrared study of the nature of bone carbonate. J. Amer. chem. Soc. 1955, **77**, 317.
28. Neuman, W. F., Toribara, T. Y., and Mulryan, B. J. The surface chemistry of bone. IX. Carbonate: phosphate exchange. J. Amer. chem. Soc. 1956, **78**, 4263.
29. Goto, K. Mineral metabolism in experimental acidosis. J. biol. Chem. 1918, **36**, 355.
30. Irving, L., and Chute, A. L. The participation of the carbonates of bone in the neutralization of ingested acid. J. cell. comp. Physiol. 1932, **2**, 157.
31. Ashby, W. Carbonic anhydrase as a factor in the organization of the central nervous system. J. nerv. ment. Dis. 1951, **114**, 391.
32. Koch, A., and Woodbury, D. M. Kinetics of cellular action of injected bicarbonate in cerebral cortex. Fed. Proc. 1957, **16**, Part I, 313.
33. Conway, E. J., and Fearon, P. J. The acid-labile CO_2 in mammalian muscle and the pH of the muscle fibre. J. Physiol. 1944, **103**, 274.
34. Meldrum, N. U., and Roughton, F. J. W. The state of carbon dioxide in blood. J. Physiol. 1933, **80**, 143.
35. Danielson, I. S., Chu, H. I., and Hastings, A. B. The pK_1' of carbonic acid in concentrated protein solutions and muscle. J. biol. Chem. 1939, **131**, 243.

CONGENITAL JAUNDICE IN RATS, DUE TO A DEFECT IN GLUCURONIDE FORMATION [1]

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It has recently been reported that bilirubin is excreted in the bile as a water-soluble glucuronide (1–3). The conjugation of bilirubin is believed to occur mainly in the liver (4, 5). *In vitro,* formation of bilirubin glucuronide has been demonstrated with a system containing liver microsomes as the enzyme source and uridine diphosphate glucuronic acid as the glucuronic acid donor (5, 6). Since conjugation of bilirubin appears to be essential for its excretion in the bile, a defect in the glucuronide forming mechanism would result in impaired pigment excretion and, hence, in retention of free bilirubin in the blood.

In preliminary communications (7, 8), such defects in glucuronide formation have been reported in congenital nonhemolytic nonobstructive jaundice in man (9) and in hereditary nonhemolytic jaundice in rats (10). The present report is concerned with the details of the murine studies, while the human syndrome will be considered in a separate communication.

MATERIALS AND METHODS

Nonjaundiced Wistar rats, known to be carriers of the jaundice trait, were donated by Dr. W. E. Castle at the University of California in Berkeley. Litters obtained by mating pairs of these animals [3] regularly included one or more offspring with jaundice. Nonjaundiced litter mates and normal Wistar and Sprague-Dawley rats served as controls. Only male animals of at least 200 Gm. body weight were used for the experiments. The rats were housed in specially built glass cages which permitted the separate collection of urine and feces (11) and were fed Purina laboratory chow.

Blood was removed from the tail or the subclavian veins for the following determinations: hemoglobin concentration (12), reticulocyte count (13), one minute direct-reacting and total serum bilirubin (14), and paper chromatographic analysis of the serum bilirubin azo derivatives (15). The urine was examined for bilirubin with the Harrison test (16) and by paper chromatographic analysis (15). Fecal urobilinogen excretion was estimated in stool samples collected over four consecutive days, and the values were expressed in mg. per day per 100 Gm. body weight (17).

o-Aminobenzoic acid and menthol were dissolved in 50 per cent ethanol and were administered by stomach tube in doses of 25 mg. per 100 Gm. body weight. During the 24 hour period immediately following the drug administration, the total urinary glucuronides were estimated (18) and expressed as glucuronic acid. For comparison, glucuronide excretion was determined during similar control periods when the rats were given only 50 per cent ethanol. Aqueous sodium *o*-aminobenzoate was injected intraperitoneally (100 mg. salt per 100 Gm. body weight) and urinary glucuronide excretion was estimated over a seven hour period immediately following the administration of the compound. The values obtained were compared with those found after injection of an equivalent amount of sodium chloride at the same hour of the preceding day. In all instances, the difference in the amount of glucuronic acid excreted during the test period and the control periods was expressed in per cent of the dose of *o*-aminobenzoic acid and of menthol administered. Sodium benzoate was injected intraperitoneally (50 mg. benzoic acid per 100 Gm. body weight) and the urinary excretion of hippurate was estimated over a 12 hour period, following the injection of the compound. The hippuric acid was extracted and crystallized (19), and the amino nitrogen of the crystals was determined by the micro-Kjeldahl method (20). Results were expressed in per cent of the dose of benzoic acid administered.

For estimation of enzymatic activity, the rats were decapitated and the livers were immediately chilled and homogenized with cold isotonic KCl. Microsomes were prepared as described previously (21). Uridine diphosphate (UDP) glucose dehydrogenase activity in the liver was

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TABLE I

Serum bilirubin concentration in Wistar rats

Rats	No.	One minute direct-reacting		Total bilirubin	
		Mean	S.D.*	Mean	S.D.
		mg.%		*mg.%*	
Jaundiced	14	0.21	0.05	8.09	2.85
Nonjaundiced litter mates	5	<0.1		<0.1	
Normal controls	5	<0.1		<0.1	

* Standard deviation.

assayed by the method of Strominger, Maxwell, Axelrod, and Kalckar (21). The rate of formation of *o*-aminophenyl glucosiduronic acid (22) was determined by incubating microsomes obtained from one Gm. of liver with 0.2 micromole of *o*-aminophenol and 0.3 micromole of UDP glucuronic acid which was prepared enzymatically (21). In the kidney, glucuronide formation was estimated by incubating tissue slices in Krebs-Ringer bicarbonate with *o*-aminophenol and UDP glucuronic acid (22).

For histological examination frozen sections were used, as well as paraffin sections of material that had been fixed in formalin or in Zenker's solution. The golden material found at the tip of the renal papilla of jaundiced animals was freed under a dissecting microscope and was dissolved in 0.2 ml. of chloroform. The azo derivatives prepared from this material were studied by paper chromatography (15).

For collection of bile, the rats were anesthetized with ether, the peritoneal cavity was opened by a midline incision, and the common bile duct was ligated in its distal portion. The dilated proximal part of the duct was then intubated with a polyethylene tube (outside diameter, 0.024 inch), which was anchored with a silk ligature. Bile flow at the rate of 0.8 to 1.0 ml. per hour was maintained by subcutaneous injection of 1.0 ml. normal saline per hour per 100 Gm. body weight. Aliquots of the bile were removed at different time intervals for estimation of bilirubin concentration (14) and for paper chromatographic analysis of the bilirubin azo derivatives (15).

Crystalline bilirubin (Armour and Company) was dissolved in 0.2 ml. of 2 per cent sodium carbonate, containing 0.05 ml. of 1 N sodium hydroxide. For intravenous injection, this solution was mixed with normal rat serum to give a final concentration of 1 mg. bilirubin per ml. As source of bilirubin glucuronide, serum was collected from patients with complete biliary obstruction, or sterile bile was obtained from patients with a T-tube in the common bile duct. Before these specimens were injected, their approximate concentration of conjugated bilirubin was estimated by the direct van den Bergh method (14) and by paper chromatography (15). Bromsulphalein (0.5 mg. per 100 Gm. body weight) was injected intravenously and the concentration of the dye in serum and bile was determined at different time intervals (23). Cholografin® (iodopamide) was injected intravenously (0.1 ml. per 100 Gm. body weight) and the excretion of the contrast medium into the duodenum was followed by serial X-rays.

TABLE II

Fecal urobilinogen excretion in Wistar rats

Rats	No.	Urobilinogen	
		Mean	S.D.
		mg./day/100 Gm. body weight	
Jaundiced	3	0.009	0.004
Nonjaundiced litter mates	3	0.039	0.013
Normal controls	4	0.077	0.024

RESULTS

A. Hematological and pigmentary studies.

Hematological studies, performed on 10 jaundiced rats, yielded a mean hemoglobin concentration of 13.2 Gm. per cent (S.D., 1.5 Gm. per cent) and a mean reticulocyte count of 0.69 per cent (S.D., 0.41 per cent). These values are within normal limits (24). The values for serum bilirubin concentration are summarized in Table I. On paper chromatographic analysis, the sera of jaundiced rats exhibited only the azo derivatives of free, nonconjugated bilirubin. In nonjaundiced litter mates and in normal control rats the plasma was colorless and bilirubin azo derivatives were not demonstrable on paper chromatography. Bilirubin could not be detected in the urine of any of the three groups of rats.

In the bile, the bilirubin concentration of five jaundiced rats was 3.32 mg. per cent (S.D., 1.04 mg. per cent). On paper chromatographic analysis, azo derivatives of bilirubin glucuronide could not be demonstrated, but there were small amounts of other azo derivatives, which exhibited Rf's similar to that of the azo derivatives of crystalline bilirubin. In addition, these bile specimens contained some yellow pigments which gave a negative van den Bergh reaction. In normal control rats and in nonjaundiced litter mates, virtually all of the pigment in the bile was present as bilirubin glucuronide.

As shown in Table II, fecal urobilinogen excretion was much smaller in jaundiced rats than in nonjaundiced animals. Patency of the biliary excretory apparatus was demonstrated by the ob-

TABLE III

Urinary excretion of glucuronic acid after administration of o-aminobenzoic acid and of menthol to Wistar rats

Rats	No.	Compound given	Dose given per 100 Gm.	Mode of administration	Glucuronic acid excreted in % of dose administered*	
					Mean or range	S.D.
			mg.			
Jaundiced	4	*o*-aminobenzoic acid	25	Oral	22.8	2.1
Nonjaundiced litter mates	9	*o*-aminobenzoic acid	25	Oral	70.1	16.4
Normal controls	4	*o*-aminobenzoic acid	25	Oral	93.2	4.8
Jaundiced	2	Sodium *o*-aminobenzoate	100	Intraperitoneal	8.4–10.9	
Nonjaundiced litter mates	4	Sodium *o*-aminobenzoate	100	Intraperitoneal	25.4	3.2
Jaundiced	2	Menthol	25	Oral	28–42	
Nonjaundiced litter mates	2	Menthol	25	Oral	70–72	

* All values are corrected for basal glucuronide excretion.

servation that Cholografin® could be recognized in the duodenum 20 minutes after its intravenous injection. Furthermore, in two jaundiced rats in which bromsulphalein had been injected intravenously the dye was cleared from the plasma in 45 minutes, and was quantitatively recovered in the bile.

In three jaundiced rats, the urinary excretion of hippurate was 75 per cent (S.D., 2.2 per cent) of the administered dose of benzoate, while in three normal control animals the corresponding value was 57 per cent (S.D., 1.7 per cent).

B. Glucuronide formation in vivo

In 8 jaundiced rats, the basal urinary excretion of glucuronic acid amounted to 4.7 mg. per day (S.D., 1.3 mg.), while 24 determinations in 11 nonjaundiced animals gave a mean value of 9.6 mg. per day (S.D., 2.1 mg.). The difference in daily glucuronic acid excretion between nonjaundiced litter mates and normal control rats was statistically not significant. As seen in Table III, the urinary excretion of glucuronic acid following oral administration of *o*-aminobenzoic acid was much smaller in jaundiced rats than in nonjaundiced controls ($p < 0.001$). Similar observations were made after oral administration of menthol and after intraperitoneal injection of sodium *o*-aminobenzoate (Table III).

C. Glucuronide formation in vitro.

Data for the *in vitro* biosynthesis of UDP glucuronic acid from UDP glucose with hepatic UDP glucose dehydrogenase are given in Table IV. Data are also presented for the formation of *o*-aminophenyl glucosiduronic acid from *o*-aminophenol and UDP glucuronic acid by liver microsomes. UDP glucose dehydrogenase was found to be about equally active in the liver of all three groups of rats. On the other hand, the rate of

TABLE IV

Enzymatic synthesis of uridine diphosphate (UDP) glucuronic acid and of o-aminophenyl glucosiduronic acid by rat liver in vitro

Rats	No.	UDP glucuronic acid formed	*o*-aminophenyl glucosiduronic acid formed	p
		μM	*μM*	
Jaundiced	6	4.9	0.008	
Nonjaundiced litter mates	8	4.3	0.034	<0.001
Sprague-Dawley controls	7	5.8	0.061	<0.01

formation of *o*-aminophenyl glucosiduronic acid by liver microsomes of jaundiced rats was much lower than that of nonjaundiced controls. A statistically significant difference was also found between nonjaundiced litter mates and normal Sprague-Dawley rats (Table IV). When equal amounts of liver microsomes from jaundiced Wistar rats and from normal Sprague-Dawley rats were mixed, no reduction in the rate of glucuronide formation of the latter was observed. With kidney slices, formation of small amounts of *o*-aminophenyl glucosiduronic acid was demonstrable in nonjaundiced rats, but none was detectable in jaundiced animals.

D. Biliary excretion of injected pigment

In Figure 1, typical data are presented on biliary bilirubin excretion in a 330 Gm. jaundiced rat, which had been given intravenous injections, first of 1.5 mg. crystalline bilirubin, and 30 minutes later of 0.8 mg. bilirubin glucuronide. The bile was collected before, during and after the injections over consecutive periods, ranging from 10 to 30 minutes. The serum bilirubin concentration, determined prior to the injections, was 8.2 mg. per cent, all of which was present in the nonconjugative form. Immediately after the injection of crystalline bilirubin, the serum bilirubin level rose to 13.5 mg. per cent but it gradually fell to 11.6 mg. per cent after 30 minutes and to 7.1 mg. per cent after 160 minutes. The injection of bilirubin glucuronide increased the 1 minute direct-reacting serum fraction temporarily from 0.2 to 1.5 mg. per cent.

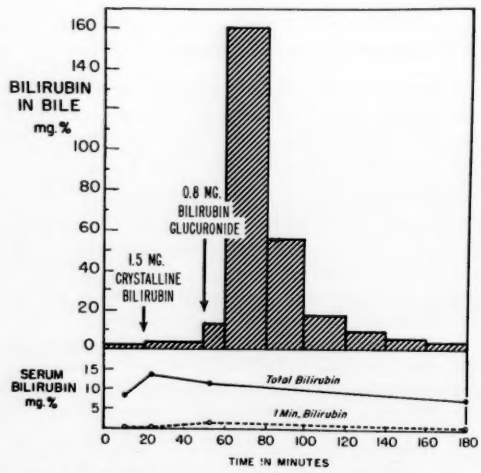

FIG. 1. BILIRUBIN EXCRETION IN THE BILE OF A JAUNDICED RAT AFTER INTRAVENOUS INJECTIONS OF BILIRUBIN

One and five-tenths mg. crystalline bilirubin and 0.8 mg. bilirubin glucuronide were injected in the tail vein of a 330 Gm. jaundiced Wistar rat. The conjugated bilirubin was rapidly excreted in the bile, while the injection of crystalline bilirubin resulted only in a slightly increased concentration of nonconjugated bilirubin.

At the beginning of the observation period, the bilirubin concentration in the bile was 1.6 mg. per cent, none of which was bilirubin glucuronide. The injection of crystalline bilirubin was followed by an only slight increase in the concentration of nonconjugated bilirubin to 2.8 mg. per cent, but no glucuronide was demonstrable. After the injection of conjugated bilirubin, on the other hand, a rapid increase in biliary pigment excretion was observed, which during the second collection period, raised the bilirubin concentration in the bile approximately one hundred fold over the preinjection level. Virtually all of the excreted pigment was present in the form of the glucuronide. At the end of a two hour period, paper chromatographic analysis indicated that all the injected bilirubin glucuronide had been cleared from the plasma and that the bile no longer contained bilirubin glucuronide.

E. Anatomical and histological findings

At necropsy of jaundiced rats, icterus was particularly evident in the peritoneal lining and in the subcutaneous and mesenteric tissues. The liver was grossly and microscopically normal, and the extrahepatic bile ducts were patent and not distended. Fecal material in the lumen of the intestinal tract was of the normal brown color. No abnormal pigmentation could be recognized in the central nervous system.

The kidneys of all adult jaundiced rats exhibited a grossly demonstrable deposit of orange material at the tip of the renal papilla. On microscopic study of frozen sections, this material was shown to consist of many rhomboid and needle-like crystals which were deposited in the interstitial tissue, and which resembled bilirubin crystals (25) (Figure 2). The tubular epithelium was not pigmented. Paper chromatographic analysis of the

azo derivatives prepared from the crystals (15) revealed them to consist of free, nonconjugated bilirubin. In some of the jaundiced rats, small numbers of similar crystals could occasionally be found in the adrenal medulla, in the brain, and in the mucosa of the intestinal tract.

Nonjaundiced litter mates and normal control rats showed no anatomical or histological abnormalities.

DISCUSSION

Malloy and Loewenstein, in studying the jaundice of this mutant strain of Wistar rats, concluded that the icterus was not hemolytic in nature (10). This is confirmed by the present investigation which revealed normal values for hemoglobin concentration (24) and reticulocyte counts and reduced fecal urobilinogen excretion. That the jaundice was not obstructive in nature was indicated by the absence of conjugated bilirubin in the plasma and of bilirubin excretion in the urine (15). Furthermore, the efficiency with which injected bromsulphalein, Cholografin® and conjugated bilirubin were excreted in the bile demonstrated that the hepatic excretory apparatus was functioning normally and that the biliary ductal system was patent.

The retention of free bilirubin in the plasma associated with reduced excretion of fecal urobilinogen was suggestive of a defect in the glucuronide forming mechanism (26). In the jaundiced rats, such a defect in glucuronide formation has been demonstrated *in vitro* and *in vivo*. *In vitro,* it was found that while UDP glucuronic acid, the glucuronic acid donor, was formed at a normal rate, the liver microsomes were deficient in the enzyme system, which transfers the glucuronic acid to suitable aglycones. As a result of this enzymatic defect, the rate of glucuronide formation in the liver was much reduced as compared to normal control rats but it was not entirely absent. The results obtained by mixing liver microsomes of jaundiced and of nonjaundiced animals, indicated that reduction in glucuronide formation was not due to the presence of enzyme inhibitors. These findings differ from a recent report by Carbone and Grodsky (27), who stated that in jaundiced rats "glucuronyl transferase activity is absent or inhibited." The presence of a defect in glucuronide formation in these jaundiced rats had recently been confirmed by Lathe and Walker (28).

In vivo, jaundiced rats were shown to excrete less glucuronic acid than nonjaundiced animals under basal conditions. Similarly, jaundiced rats responded to loading doses of aglycones with smaller increases in glucuronic acid excretion than normal animals and nonjaundiced litter mates.

In view of these findings, one might have anticipated that the formation of bilirubin glucuronide would proceed at a similarly reduced rate, but would not be entirely absent. It was therefore surprising to find that on paper chromatographic analysis, bile from jaundiced rats consistently failed to reveal even trace amounts of bilirubin glucuronide. On the other hand, small amounts of nonconjugated bilirubin were regularly demonstrable in such specimens.

Two explanations are offered to account for these observations. Since it appears that a single enzyme system is involved in all types of glucuronides formed (29), it is possible that bilirubin is unsuccessful in competing with other normally occurring aglycones for the deficient glucuronic acid transferase system (8). If this hypothesis is correct, it must be assumed that small amounts of free bilirubin probably attached to proteins are capable of leaking from the extracellular fluid into the bile. Another and more likely explanation is

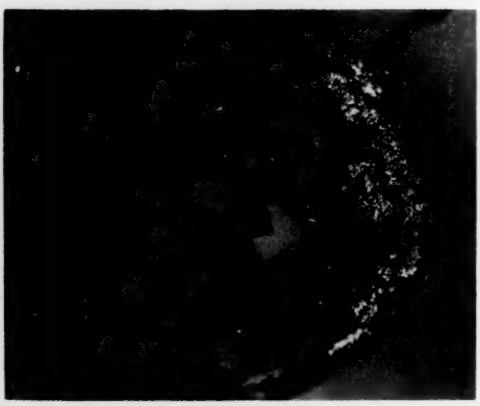

FIG. 2. BILIRUBIN IN THE RENAL PAPILLA OF A JAUNDICED RAT

Frozen section, photographed with polarized light at partial extinction. The light areas represent bilirubin crystals, the dark ones amorphous precipitates of bilirubin (magnification times 75).

that in jaundiced rats, small amounts of bilirubin glucuronide are actually formed, but are rapidly hydrolyzed after their excretion into the bile, and thus escape chromatographic identification.

Regardless of which of these two explanations is correct, it is evident that in the absence of an efficiently functioning mechanism for glucuronide formation, biliary bilirubin excretion is severely impaired. This was apparent from the lower bilirubin concentration in the bile and the reduced urobilinogen excretion in the feces of jaundiced rats as compared to normal rats (30). More important, however, is the observation that in jaundiced rats injected bilirubin glucuronide was rapidly excreted in the bile, whereas free bilirubin was not. The intravenous administration of crystalline bilirubin to jaundiced rats resulted in the excretion of less than 1 per cent of the amount of pigment that was excreted by normal rats with a comparable serum bilirubin level (31). On the other hand, injection of conjugated bilirubin was followed by a more than one hundred fold increase in biliary pigment excretion. The maximal clearance for bilirubin glucuronide in jaundiced rats was similar to the maximal clearance for crystalline bilirubin in normal rats as determined by Weinbren and Billing (31).

In the mammalian organism, most metabolites which are excreted in conjugated form can combine with more than one hydrophilic group (32, 33). The finding that in jaundiced rats a larger fraction of the injected benzoate was converted to hippurate than in normal rats, is believed to indicate that the defect in glucuronide formation may in part be compensated by activation of other detoxication mechanisms. In the present study, however, no such compensatory mechanism has been demonstrated for the conjugation of bilirubin. Although normal rat bile regularly contained small amounts of water-soluble, diazo-positive pigments of a nonglucuronide nature, this pigment fraction was not increased in jaundiced rats. It is surprising that in spite of the severe impairment in pigment excretion, the physiological turnover of heme compounds did not result in a steadily increasing degree of jaundice. It would appear that alternative pathways of heme metabolism must exist to account for the lack of progressivly increasing icterus in these animals (4). This may also explain the observation that after the injection of crystalline bilirubin to jaundiced rats (Figure 1), the concentration of free bilirubin in the serum decreased much more rapidly than could be accounted for by the excretion of pigment in the bile. It is realized, however, that this fall in serum bilirubin concentration may in part have been due to redistribution of pigment in the extravascular space (34).

Since breeding of nonjaundiced litter mates produced offspring which were in part jaundiced, it may be assumed that such rats were carriers of the jaundice trait. Although their serum bilirubin levels were not elevated, their rate of glucuronide formation was reduced as compared to normal Wistar and Sprague-Dawley rats, but it was greater than in jaundiced animals. This would suggest that in nonjaundiced litter mates the metabolic abnormality is less severe than in jaundiced rats and is insufficient to produce pigment retention in the serum. Since it has been observed that significant differences in glucuronide metabolism exist between the two sexes in rats (35), only male animals have been used in the present study. Because of this limitation and the relatively small number of animals studied, the available data are believed to be insufficient to establish the genetic pattern of this disorder.

In the plasma, nonconjugated bilirubin is attached to proteins, mainly albumin (36), which keeps it in solution, since at physiological pH it is almost insoluble in water (37). In the tissues, on the other hand, information is lacking as to whether bilirubin is attached to extravascular serum albumin or to other tissue proteins. The presence of bilirubin crystals in the renal papilla and in other organs of jaundiced rats seems to indicate that the local concentration of bilirubin must have exceeded the capacity to bind the pigment. It is of interest to note that in conditions associated with high concentrations of nonconjugated bilirubin in man, bilirubin crystals in tissues have also been observed (25). In three patients with congenital nonhemolytic jaundice (9), which is believed to be due to a defect in glucuronide formation (7, 8), Smith, Wheeler, Bauer, and Saltzstein have found bilirubin crystals in the renal papillae (38). The significance of these observations must be clarified by further studies.

SUMMARY

1. Wistar rats of a mutant strain, exhibiting congenital jaundice, were studied. The icterus was shown to be nonhemolytic and nonobstructive in nature.

2. In jaundiced rats, no bilirubin glucuronide was demonstrable in serum, bile or urine. The bile contained small amounts of free bilirubin. Fecal urobilinogen excretion was greatly reduced.

3. The liver was histologically normal, and liver function tests, such as hippuric acid formation and excretion of bromsulphalein, Cholografin® and conjugated bilirubin were normal.

4. *In vivo* and *in vitro*, marked impairment in glucuronide formation was demonstrated in jaundiced rats, due to a defect in the glucuronyl transferase system. Since conjugation of bilirubin appears to be essential for its excretion in the bile, this enzymatic defect in glucuronide formation is believed to be the cause for the impaired pigment excretion and the retention of free bilirubin in the plasma.

5. Nonjaundiced litter mates of icteric rats were found to have a reduced ability to form glucuronides, but the defect was less severe than in jaundiced animals and did not result in retention of bilirubin in the plasma.

6. Crystals consisting of nonconjugated bilirubin were observed in the renal papilla and in other organs of jaundiced rats.

REFERENCES

1. Schmid, R. Direct-reacting bilirubin, bilirubin glucuronide, in serum, bile and urine. Science 1956, **124**, 76.
2. Billing, B. H., and Lathe, G. H. The excretion of bilirubin as an ester glucuronide, giving the direct van den Bergh reaction. Biochem. J. 1956, **63**, 6 p.
3. Talafant, E. Properties and composition of the bile pigment, giving a direct diazo reaction. Nature (Lond.) 1956, **178**, 312.
4. Schmid, R. Some aspects of bile pigment metabolism. Clin. Chem. 1957, **3**, 394.
5. Billing, B. H., and Lathe, G. H. Bilirubin metabolism in jaundice. Amer. J. Med. 1958, **24**, 111.
6. Schmid, R., Hammaker, L., and Axelrod, J. The enzymatic formation of bilirubin glucuronide. Arch. Biochem. 1957, **70**, 285.
7. Schmid, R., Axelrod, J., Hammaker, L., and Rosenthal, I. M. Congenital defects in bilirubin metabolism (abstract). J. clin. Invest. 1957, **36**, 927.
8. Axelrod, J., Schmid, R., and Hammaker, L. A biochemical lesion in congenital, non-obstructive, nonhaemolytic jaundice. Nature (Lond.) 1957, **180**, 1426.
9. Crigler, J. F., Jr., and Najjar, V. A. Congenital familial nonhemolytic jaundice with kernicterus. Pediatrics 1952, **10**, 169.
10. Malloy, H. T., and Loewenstein, L. Hereditary jaundice in rat. Canad. med. Ass. J. 1940, **42**, 122.
11. Draper, H. H., and Robbins, A. F. A urinofecal separator for laboratory rat. Proc. Soc. exp. Biol. (N. Y.) 1956, **91**, 174.
12. Sunderman, F. W., MacFate, R. P., MacFayden, D. A., Stevenson, G. F., and Copeland, B. E. Clinical Hemoglobinometry. Baltimore, The Williams and Wilkins Company, 1953.
13. Brecher, G. New methylene blue as a reticulocyte stain. Amer. J. clin. Path. 1949, **19**, 895.
14. Ducci, H., and Watson, C. J. The quantitative determination of the serum bilirubin with special reference to the prompt-reacting and the chloroform-soluble types. J. Lab. clin. Med. 1945, **30**, 293.
15. Schmid, R. The identification of "direct-reacting" bilirubin as bilirubin glucuronide. J. biol. Chem. 1957, **229**, 881.
16. Hawkinson, V., Watson, C. J., and Turner, R. H. Modification of Harrison's test for bilirubin in the urine especially suited for mass and serial use. J. Amer. med. Ass. 1945, **129**, 514.
17. Schwartz, S., Sborov, V., and Watson, C. J. Studies of urobilinogen. IV. The quantitative determination of urobilinogen by means of the Evelyn photoelectric colorimeter. Amer. J. clin. Path. 1944, **14**, 598.
18. Dische, Z. A new specific color reaction of hexuronic acids. J. biol. Chem. 1947, **167**, 189.
19. Quick, A. J. The study of benzoic acid conjugation in the dog with a direct quantitative method for hippuric acid. J. biol. Chem. 1926, **67**, 477.
20. Ma, T. S., and Zuazaga, G. Micro-Kjeldahl determination of nitrogen. Industr. engin. Chem. (Analyt. Ed.) 1942, **14**, 280.
21. Strominger, J. L., Maxwell, E. S., Axelrod, J., and Kalckar, H. M. Enzymatic formation of uridine diphosphoglucuronic acid. J. biol. Chem. 1957, **224**, 79.
22. Levvy, G. A., and Storey, I. D. E. The measurement of glucuronide synthesis by tissue preparations. Biochem. J. 1949, **44**, 295.
23. Gaebler, O. H. Determination of bromsulphalein in normal, turbid, hemolyzed or icteric serums. Amer. J. clin. Path. 1945, **15**, 452.
24. Wintrobe, M. M. Clinical Hematology, 3rd ed. Philadelphia, Lea and Febiger, 1951.
25. Dunn, T. B. Hematoidin crystals in reticulum cell sarcoma of the mouse and in newborn human tissues. Milit. Surg. 1951, **109**, 350.

26. Schmid, R. Neuere Gesichtspunkte auf dem Gebiete des Gallenfarbstoffwechsels. Helv. med. Acta 1957, **24**, 273.
27. Carbone, J. V., and Grodsky, G. M. Constitutional nonhemolytic hyperbilirubinemia in the rat: Defect of bilirubin conjugation. Proc. Soc. exp. Biol. (N. Y.) 1957, **94**, 461.
28. Lathe, G. H., and Walker, M. An enzymatic defect in human neonatal jaundice and in Gunn's strain of jaundiced rats. Biochem. J. 1957, **67**, 9 p.
29. Axelrod, J., Inscoe, J., and Tomkins, G. M. The enzymatic formation of N-glucuronocyl acids. J. biol. Chem. In press.
30. Hanzon, V. Liver cell secretion under normal and pathologic conditions studied by fluorescence microscopy on living rats. Acta physiol. scand. 1952, **28**, Suppl. **101**.
31. Weinbren, K., and Billing, B. H. Hepatic clearance of bilirubin as an index of cellular function in the regenerating rat liver. Brit. J. exp. Path. 1956, **37**, 199.
32. Williams, R. T. Detoxication Mechanisms. New York, John Wiley and Sons, 1947.
33. Quick, A. J. The conjugation of benzoic acid in man. J. biol. Chem. 1931, **92**, 65.
34. Dragstedt, C. A., and Mills, M. A. The removal of intravenously injected bilirubin from the blood stream in the dog. Amer. J. Physiol. 1937, **119**, 713.
35. Axelrod, J. Unpublished observations.
36. Klatskin, G., and Bungards, L. Bilirubin-protein linkages in serum and their relation to the van den Bergh reaction. J. clin. Invest. 1956, **35**, 537.
37. Overbeek, J. T. G., Vink, G. L. J., and Deenstra, H. The solubility of bilirubin. Rec. Trav. chim. Pays-Bas 1955, **74**, 81.
38. Smith, M. G., Wheeler, J. D., Bauer, W. C., and Saltzstein, S. L. Personal communication.

SALICYLATES AND THYROID FUNCTION. I. DEPRESSION OF THYROID FUNCTION

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In 1916 Denis and Means (1) first observed that salicylate administration increased oxygen consumption and carbon dioxide production in man. This finding has since been confirmed both in man (2, 3) and in experimental animals (4–6). Although salicylates increase the resting minute ventilation, it has been demonstrated that the elevation in basal metabolic rate is not dependent on an increase in respiratory work. Decapitated or curarized dogs maintained in a total body respirator to keep ventilation constant showed the same increased oxygen consumption in response to salicylate administration as the intact animal (5). Furthermore, oxygen consumption has been shown to be increased in tissues removed from salicylate-treated animals (7) and in isolated tissue or mitochondrial preparations to which salicylates have been added (8, 9). The increase in oxygen consumption is accompanied by a reduction in the esterification of inorganic phosphate, *i.e.*, salicylates uncouple oxidative phosphorylation (7).

The administration of 2,4-dinitrophenol to man or animals leads to strikingly similar alterations. Basal metabolic rate is increased in the intact animal (10–12), tissue oxygen consumption is stimulated in slices (13), and oxidative phosphorylation is uncoupled in isolated mitochondrial preparations (14, 15). Indeed, several authors have commented on the similarity of 2,4-dinitrophenol and salicylate (6, 8, 16).

It has recently been shown that 2,4-dinitrophenol produces a generalized depression of thyroid function in rats (13, 17, 18) and reduces the serum protein bound iodine concentration (PBI) in rats (17) and man (19). The toxicity of 2,4-dinitrophenol (12) precludes a thorough study of its effect on thyroid function in man. Salicylates, on the other hand, are well suited for such an investigation and preliminary studies (20) showed that salicylate administration increased the metabolic rate while simultaneously depressing thyroid function.

MATERIAL AND METHODS

The effect of salicylate administration on thyroid function was studied in 25 male patients ranging in age from 18 to 38 years. Twenty had rheumatoid arthritis; and five were hospitalized for other conditions—myxedema, gout, acne, and two for chronic otitis media. Balance studies were performed in a metabolic unit. Nineteen patients participated in studies of the effect of chronic salicylate therapy and received salicylates for 8 to 15 weeks, and six took part in acute studies during which salicylates were given for 7 to 10 days. No other medications except an occasional sedative were permitted; daily physical therapy was encouraged. Six of the patients participating in the chronic studies had an elevated erythrocyte sedimentation rate at the beginning of the study.

Enteric coated sodium salicylate was given every four hours. In long term studies the initial dose of 6.0 Gm. per day was gradually increased, reaching 8.1 Gm. in certain instances. In the acute experiments 9.0 Gm. was administered daily throughout the study. Serum "salicyl" (salicylate and salicylurate) concentration (21) was measured at weekly intervals, or more frequently if the study was short.

The basal metabolic rate was determined three or more times before and/or after salicylate administration, at weekly intervals during chronic salicylate therapy, and more frequently during acute experiments. The patients were fasted 12 hours and slept in a secluded room. Oxygen consumption was measured using the closed circuit technique and basal metabolic rate was obtained from factor tables.[1]

Twenty-four hour urine samples were collected for creatinine and creatine determinations (22).[2] Standard

[1] Operation Manual for Model 10 Sanborn Metabulator, 1952.

[2] It was found that the addition of salicylate or gentisate to urine in amounts approximating their estimated urine concentration significantly increased the apparent urine creatine value. Since the false positive effect of gentisate was 30 to 40 times as great as that of salicylate on a molar basis and the conversion to gentisate ranges

methods were employed for the measurement of serum cholesterol (25, 26). The addition of salicylate to normal sera to a concentration of 45 mg. per 100 ml. did not interfere with the protein bound iodine determination by the method of Barker, Humphrey, and Soley (27).[3]

In the studies of thyroxine disappearance, I^{131}-labeled L-thyroxine [4] was injected intravenously in doses ranging from 25 to 75 microcuries with a specific activity of 14 to 29 microcuries per microgram. Prior to injection the radiothyroxine was diluted with human serum albumin [5] to diminish absorption on the syringe and glassware used to prepare the standard. Serum samples collected 10 minutes after injection and each morning were counted in a well-type scintillation counter. When observations were desired for more than 15 days, counting was done with a specially shielded differential pulse height analyzer. Slopes were determined on the basis of a minimum of eight daily points. Half-life, turnover rate, pool size, and degradation rate were calculated as by Sterling, Lashof, and Man (28). Thyroxine binding by serum protein was measured by the reverse flow zone electrophoresis method of Robbins (29).

The radioiodine uptake was determined following an oral tracer dose of 7.5 to 10 microcuries of carrier-free I^{131}. The thyroid region was counted at 2, 5 and 24 hours with a wide angle scintillation counter at a distance of 15 cm.; body background was estimated by counting over the thigh. No subject received more than a cumulative total of 125 microcuries of I^{131}.

During the measurement of thyroid and renal clearance of radioiodine urine flow was maintained by sustained water diuresis at 8 to 15 ml. per minute to minimize urinary dead space and facilitate complete collections. Urine was obtained by spontaneous voiding 35 minutes after injection of radioiodine and every half hour thereafter. Blood was collected through an indwelling brachial vein catheter during the second and fifteenth minute and, subsequently, at half-hour intervals from the time of radioiodine injection. The thyroid region was counted each minute during the first five minutes and, thereafter, for a five minute period at half-hour intervals from the time of injection. Samples of plasma, urine, and an aliquot of the administered radioiodine were counted together at the end of the experiment. A plot of the logarithm of plasma radioactivity against time yielded a straight line 30 to 45 minutes after injection.

The renal clearance of radioiodine at half-hour intervals was computed using the mean plasma radioactivity as described by Keating and co-workers (30). The thyroid clearance was calculated from the rate of accumulation of radioiodine during a given interval divided by the mean plasma radioactivity. The neck counts were corrected for extrathyroidal radioactivity by assuming that all the neck radioactivity at two minutes was extrathyroidal and that it decreased proportionally to the falling plasma radioactivity. From the ratio of the neck to plasma radioactivity during the second minute and the plasma radioactivity at any subsequent time, the extrathyroidal radioactivity at that time was estimated. Thus, the thyroidal clearance of iodide in ml. per minute is:

$$C\,I^{131} = \frac{N_2 - N_1}{T \times P},$$

where N equals corrected neck counts per minute at times t_1 and t_2; P equals plasma counts per minute per ml. calculated as the mean of a function between t_1 and t_2 (30); and T equals time interval in minutes between t_1 and t_2.

The effect of salicylate on the iodide concentrating mechanism of the thyroid was studied according to standard procedure (18). Male Sprague-Dawley rats maintained on a low iodine diet for three to six weeks were given an intraperitoneal injection of 10 mg. of 1-methyl-2-mercaptoimidazole. Forty-five minutes later 30 to 50 mg. of sodium salicylate or 20 mg. of sodium thiocyanate was injected, followed in 20 minutes by 1 microcurie of carrier-free I^{131}. The rats were sacrificed one hour later, and the radioactivity of the thyroid gland and plasma was determined.

Thyroidal organification of iodide was studied *in vitro* (31) using thyroids from male Sprague-Dawley rats kept on a low iodine diet for 26 days. Thyroid lobes were sectioned free hand and incubated under an atmosphere of oxygen in Krebs-Ringer phosphate medium, pH 7.4. Known concentrations of 1-methyl-2-mercaptoimidazole or sodium salicylate and carrier-free I^{131} were added to the medium. After three hours the slices were blotted, homogenized in cold 10 per cent trichloroacetic acid, and washed twice with cold 5 per cent trichloroacetic acid. Radioactivity of the precipitate ("organic I^{131}") and of an aliquot of the combined supernatant fractions (trichloroacetic acid soluble fraction) was determined. The combined radioactivity constituted total "uptake."

RESULTS AND DISCUSSION

The increase in oxygen consumption produced by salicylate administration (*cf.* below) was not accompanied by an elevation in the rectal temperature or a complaint of heat intolerance, although on questioning some patients were aware of increased sweating. Hyperkinesis and emotional lability were lacking, and, in fact, several patients became lethargic and slept much of the time. Neither the waking nor the sleeping pulse rate increased during the acute or protracted studies. The patient with myxedema experienced a rise in basal metabolic rate from minus 35 to plus 35 per

from 1 to 8 per cent (23, 24) accurate correction to obtain true creatine values could not be made. Nevertheless, the estimated error, assuming a 10 per cent conversion, did not account for the creatinuria observed in most patients.

[3] Performed by the Bioscience Laboratories, Los Angeles, Calif.

[4] Supplied by Abbott Laboratories, Oak Ridge, Tenn.

[5] Supplied by Armour Laboratories, Chicago, Ill.

cent without an increment in pulse rate. Similarly, in two thyrotoxic patients with a tachycardia while sleeping, salicylates failed to increase the pulse rate while further elevating the basal metabolic rate.

The mean serum salicylate level in 16 patients receiving salicylates for 8 to 15 weeks was 37 mg. per 100 ml., ranging from 22 to 47 mg. per 100 ml. In two other patients, C. M. and L. E., the concentration was maintained at 65 and 58 mg. per cent, respectively. One-third of the patients receiving salicylates chronically complained of tinnitus or diminished auditory acuity early in the treatment period, but soon became adapted and tolerated even larger doses. None of the group receiving salicylates for a protracted period was troubled by nausea or vomiting, and there were no instances of a bleeding tendency. A twofold increase in resting minute ventilation occurred in Patients C.M. and L. E., and in two other patients, K. C. and W. C., whose serum salicylate concentration exceeded 50 mg. per 100 ml. near the end of the study period. The serum bicarbonate was reduced 5 to 7 mEq. per liter in these patients, but the venous pH was not predictable, being normal, slightly elevated, or slightly depressed. The remaining patients on chronic salicylate therapy showed a mean increase of 65 per cent in resting minute ventilation, 9 per cent in respiratory rate, and 52 per cent in tidal volume, and they maintained their serum bicarbonate concentration and pH within the normal range.

Oxygen consumption

The average individual increase in oxygen consumption ranged from 41 to 104 ml. per min. per 1.73 M.², and the mean increase for the group was 73 ml. per min. per 1.73 M.² (Table I). This is equivalent to a mean rise in basal metabolic rate from a level of minus 8 to plus 22 per cent. The elevation in oxygen consumption appeared during the first 24 hours of salicylate therapy. This was documented in seven subjects in whom the increase in oxygen consumption ranged from 42 to 130 ml. per min. per 1.73 M.² after 24 hours.

The relation of the increase in oxygen consumption to the serum salicylate concentration in 16 patients on chronic salicylate therapy is depicted in Figure 1. The coefficient of correlation (r) is 0.72 ($p < 0.01$). A similar relationship has been observed in rabbits (6) and in patients with myxedema (32). The two patients, C. M. and L. E., whose mean salicylate concentrations were 65 and 58 mg. per 100 ml., respectively, were lethargic and failed to show a rise in oxygen consumption proportional to their serum salicylate concentration; their points lie to the right of the regression line shown in Figure 1. In contrast,

TABLE I

The effect of chronic salicylate administration on oxygen consumption

Mean oxygen consumption in ml. per min. per 1.73 M.²		
Control (12)*	Salicylate administration (16)	Postcontrol (15)
221 ± 16†	294 ± 23	222 ± 16

* Figures in parentheses refer to number of patients studied. The mean serum salicylate concentration was 37 ± 7 mg. per 100 ml. (mean plus or minus standard deviation).

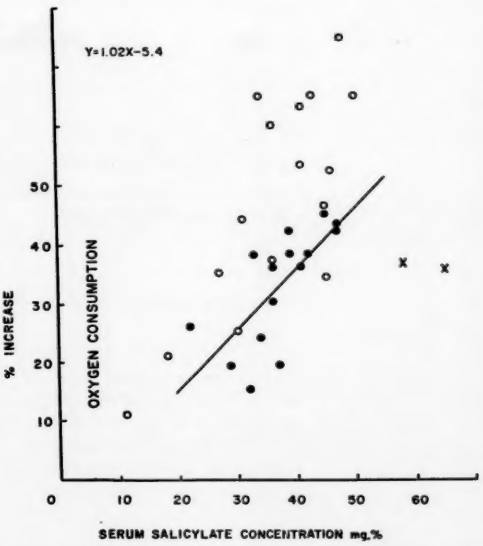

FIG. 1. CORRELATION OF OXYGEN CONSUMPTION AND SERUM SALICYLATE CONCENTRATION

The solid circles represent the mean values during the entire period of salicylate administration, and the regression line is the best fit line through these points by the method of least squares. The coefficient of correlation (r) is 0.72 ($p < 0.01$). The open circles represent single values obtained during the first 3 to 10 days of salicylate administration. The crosses illustrate the findings in Patients C. M. and L. E. (see text).

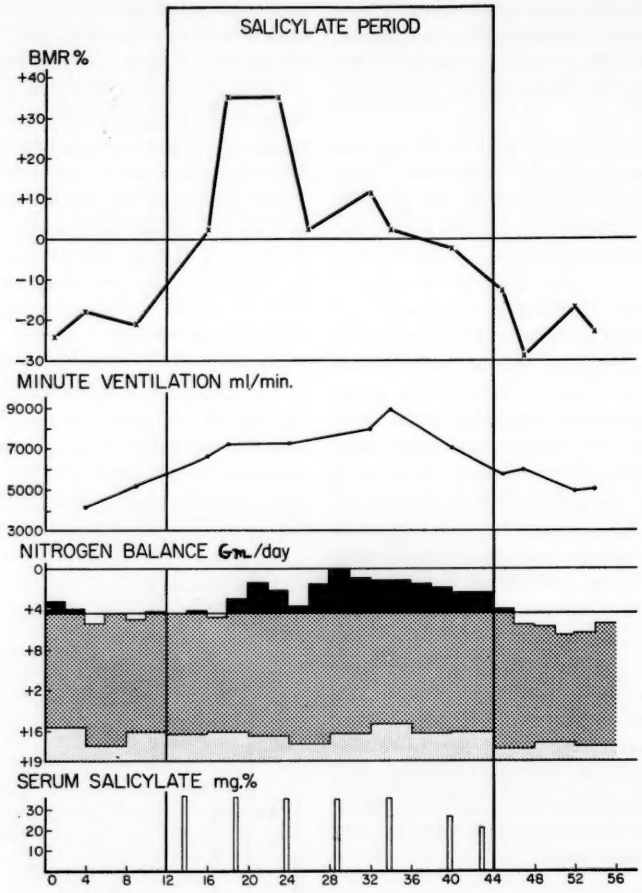

FIG. 2. EFFECT OF SALICYLATE ADMINISTRATION IN MYXEDEMA

The increase in basal metabolic rate falls off somewhat after the first 10 days although the serum salicylate concentration remains unchanged. The rise in BMR is not dependent on the increase in resting minute ventilation. The light hatching represents stool nitrogen; the black area indicates the reduction in nitrogen retention during salicylate administration as compared to the average nitrogen retention of the control period.

the metabolic stimulation for a given serum salicylate concentration was greater during the first 3 to 10 days than during subsequent chronic administration and points obtained during this period fall to the left of the regression line. This suggests some tolerance to the metabolic stimulation of salicylates.

The association of the salicylate-induced increase in oxygen consumption with a reduction in nitrogen retention was observed in several patients. Nitrogen retention was diminished 2.0 Gm. per day as compared to the control period in the patient with myxedema, and the retention in the postsalicylate period exceeded that of the control period (Figure 2). Patients L. E. and C. M. experienced a negative nitrogen balance of 16 and 22 Gm. and a weight loss of 5 and 12 pounds, respectively, during 32 days of salicylate administra-

tion. Similar results have also been obtained in other euthyroid patients maintained at lesser serum salicylate levels (33).

Cholesterol

A consistent reduction in the total serum cholesterol was noted in only 2 of 16 patients in whom the cholesterol was followed weekly during chronic salicylate administration, and in these 2 patients the decrease was only 50 mg. per 100 ml. compared to the pre- and postsalicylate administration values. In the patient with myxedema the serum cholesterol fell gradually from 240 to 160 mg. per 100 ml. in 32 days and rose when salicylate administration was discontinued. Alexander and Johnson (34) have also observed a fall in serum cholesterol in three patients with myxedema during 16 days of salicylate treatment. The failure to induce a fall in the serum cholesterol of the euthyroid patients is similar to the findings obtained with 2,4-dinitrophenol (10). Furthermore, other workers (35) have shown that although thyroid is partially effective against the hypercholesterolemia induced by cholesterol feeding in the chick, dinitrophenol has little or no effect on the plasma or tissue levels of cholesterol and offers no protection against the cholesterol-induced atherosclerosis.

Protein bound iodine (PBI) concentration

A reduction in the PBI was observed in every patient during salicylate administration (Table II), and the mean decrease was about 30 per cent of the initial value. The maximum reduction in PBI was generally achieved during the first week. No further diminution was obtained by continuing salicylate administration for 8 to 15 weeks or by increasing the maintenance dose from 7.2 to 9.0 Gm. per day. Correlation of the decrease in PBI with the serum salicylate concentration or the mean increase in oxygen consumption is not statistically significant ($r = 0.34$ and 0.32, respectively, $p > 0.10$). The fall in PBI produced by salicylate administration is similar to that observed in the rat (17) and in man (19) after 24 and 48 hours of 2,4-dinitrophenol treatment, respectively.

Radiothyroxine

The reduction in PBI concomitant with salicylate administration could result from either a decrease in production or an increase in peripheral degradation of thyroid hormone, or both. The observation that dinitrophenol diminished the PBI in a thyroidectomized patient receiving desiccated thyroid (19) suggested that increased peripheral utilization might be an important factor. Earlier studies with dinitrophenol in the rat had shown that this drug caused an increase in the fractional rate of disappearance of radiothyroxine from the circulation (18).

The effect of chronic salicylate therapy on the fractional rate of thyroxine disappearance and degradation was studied in 15 patients (Table III, a, b and c). The average half-life of radiothyroxine was 6.2 and 6.4 days during the pre- and postsalicylate period, respectively, and fell to 4.7 days during salicylate administration. As a consequence, the turnover rate [6] rose from 11.3 per cent per day in the precontrol period to 15.1 per cent per day during salicylate treatment and

TABLE II

The effect of chronic salicylate administration on the serum protein bound iodine (PBI)

Patient	Control PBI	Salicylate period PBI		Post-control PBI
		Fourth week	Ninth week	
	μg.%	μg.%		μg.%
K. C.	5.5	4.1	4.1	5.4
R. P.	5.9	3.7	3.7	5.8
F. C.	5.6	3.7	3.1	4.8
W. C.	5.0	3.6	3.2	4.5
J. B.	6.2	4.5	4.6	6.2
R. B.	5.7	4.4	5.0	
F. K.	4.9	3.1		5.4
C. M.	4.8	3.4		4.6
T. K.	6.8	3.9	4.5	7.0
L. E.	5.1	3.7		6.4
R. Pi.	4.7	4.0	3.7	4.5
C. C.		3.7	3.3	5.4
J. L.		3.3	3.9	5.0
G. D.		3.9	4.4	5.6
Mean	5.5	3.8	4.0	5.4
Standard deviation	0.6	0.3	0.6	0.7

[6] Turnover rate is here defined as the fractional rate of renewal of the extrathyroidal organic iodine pool (EOI) per unit time. In calculating turnover rate it was assumed that the PBI did not change during the experimental period, that PBI is equivalent to thyroxine, and that the serum radioactivity represented thyroxine. PBI determinations before and after the experimental period and chromatograms of the serum supported the first and last of these assumptions.

TABLE IIIA

Radiothyroxine disappearance in patients with rheumatoid arthritis prior to salicylate administration

Subject	Weight	PBI	Kinetics of disappearance*		Extrathyroidal organic iodine pool (EOI)	Degradation rate
			t 1/2	k		
	Kg.	*μg. %*	*days*	*%/day*	*μg./1.73 M.²*	*μg./1.73 M.²/day*
W. C.	58.2	5.0	5.9	11.8	516	61
J. B.†	81.4	6.6	6.2	11.2	732	82
R. B.†	57.2	6.0	6.6	10.5	696	73
F. K.	79.5	4.5	7.3	9.5	508	48
C. M.	87.3	5.0	5.6	12.4	548	68
T. K.†	53.6	6.9	5.9	11.8	700	83
L. E.	63.6	5.2	6.0	11.6	590	68
R. Pi.	73.6	4.9	6.1	11.4	497	57
Mean		5.5	6.2	11.3	598	68
Standard deviation		0.8	0.5	0.8	90	11
Mean (controls)‡		5.8	6.8	10.4	653	67
Standard deviation		0.7	1.0	1.3	69	6

* $T_{\frac{1}{2}}$ is half-time in days; k (turnover rate) is the fractional rate of renewal of the extrathyroidal organic iodine pool (EOI) per unit time.

† Erythrocyte sedimentation rate (ESR) elevated at the time the radiothyroxine disappearance study was performed.

‡ Observations on radiothyroxine disappearance in nine hospital patients admitted for conditions other than rheumatoid arthritis—four with acne, two with chronic otitis media, two with minimal renal disease, and one with peripheral vascular disease.

returned to 11.0 per cent per day in the post-salicylate period. These findings approximate the accelerated fractional disappearance rate observed in thyrotoxicosis (36, 37). Moreover, the change in radiothyroxine disappearance is correlated with the increase in oxygen consumption ($r = 0.60$, $p < 0.01$) (Figure 3). Thus, the greater the increment in oxygen consumption the greater the increase in fractional rate of radiothyroxine disappearance from the circulation.

TABLE IIIB

Radiothyroxine disappearance in patients with rheumatoid arthritis during salicylate administration *

Subject	Weight	PBI	Kinetics of disappearance		Extrathyroidal organic idoine pool (EOI)	Degradation rate
			t 1/2	k		
	Kg.	*μg. %*	*days*	*%/day*	*μg./1.73 M.²*	*μg./1.73 M.²/day*
K. C.†	77.7	4.4	5.1	13.6	455	62
R. P.	76.8	3.9	5.3	13.1	502	66
C. C.	74.1	3.7	4.0	17.3	378	65
F. C.	76.4	3.1	3.4	20.4	317	65
J. B.†	83.2	4.6	5.0	13.9	566	79
R. B.	58.2	4.7	4.9	14.2	474	67
F. K.	81.8	3.1	4.3	16.1	320	52
W. C.	65.9	3.4	4.4	15.8	399	63
C. M.	84.5	3.4	4.3	16.1	330	53
T. K.†	57.7	4.2	4.0	17.3	479	83
J. L.	72.3	3.2	4.4	15.8	363	57
L. E.	61.4	3.7	5.0	13.9	325	45
R. Pi.	75.9	4.0	5.0	13.9	444	62
D. K.†	64.1	5.1	5.1	13.6	619	84
G. D.†	68.6	4.5	6.3	11.0	632	70
Mean		3.9	4.7	15.1	440	65
Standard deviation		0.6	0.7	2.2	103	10

* Radiothyroxine disappearance was measured after the PBI had fallen to a relatively constant value, *i.e.*, after at least two weeks of salicylate administration and generally about the fourth week.

† ESR elevated at the time of the radiothyroxine study.

TABLE IIIC

Radiothyroxine disappearance in patients with rheumatoid arthritis after discontinuation of salicylate administration *

Subject	Weight	PBI	Kinetics of disappearance t 1/2	Kinetics of disappearance k	Extrathyroidal organic iodine pool (EOI)	Degradation rate
	Kg.	*μg. %*	*days*	*%/day*	*μg./1.73 M.²*	*μg./1.73 M.²/day*
K. C.†	71.8	5.3	6.6	10.5	545	57
R. P.	75.0	5.6	7.1	9.8	659	65
C. C.	74.1	5.4	7.5	9.2	510	47
F. C.	78.2	5.6	5.8	12.0	532	64
W. C.‡	59.1	4.5	5.9	11.8	487	57
J. B.‡	81.8	6.2	6.6	10.5	711	75
F. K.‡	82.7	5.4	6.0	11.6	550	64
C. M.‡	84.0	4.3	5.4	12.8	493	63
J. L.	74.1	4.8	6.0	11.6	505	59
L. E.‡	64.5	6.4	6.7	10.3	726	75
D. K.	70.5	5.6	6.3	11.0	600	66
G. D.	75.9	5.6	6.5	10.7	640	68
Mean		5.4	6.4	11.0	580	63
Standard deviation		0.6	0.5	1.0	80	8

* Radiothyroxine disappearance was measured two to four weeks after discontinuation of salicylate administration.
† ESR elevated at time of radiothyroxine study.
‡ Patients in whom radiothyroxine disappearance was studied both before (Table IIIa) and after salicylate administration.

The radiothyroxine distribution space showed no consistent change with chronic salicylate therapy, remaining unchanged in most patients, and increasing or decreasing slightly in an equal number of instances. Since the PBI concentration fell with salicylate treatment, the extrathyroidal organic iodine pool (EOI) was reduced to a mean of 440 micrograms from mean pre- and postsalicylate values of 598 and 581 micrograms, respectively. The degradation rate, being the product of the smaller EOI pool and the faster turnover rate, was found to be the same during salicylate administration as during the control periods, *i.e.*, 67 micrograms per day per 1.73 M.² before salicylates, 65 micrograms per day per 1.73 M.² during salicylates, and 63 micrograms per day per 1.73 M.² after salicylate therapy.

To determine how quickly salicylate treatment increased the fractional rate of disappearance of radiothyroxine, the effect of acute salicylate administration was studied in four euthyroid patients and a fifth with myxedema seven days after injection of radiothyroxine. An increase in the fractional rate of disappearance of radiothyroxine from the circulation occurred within the first 24 hours of salicylate administration. However, it was not possible to calculate the degradation rate because steady state conditions did not prevail,

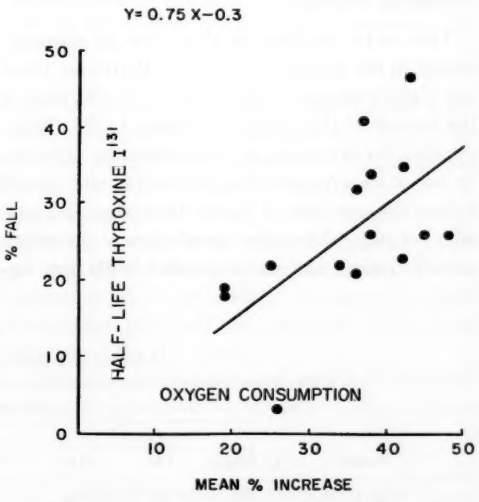

FIG. 3. CORRELATION OF OXYGEN CONSUMPTION AND THE FRACTIONAL RATE OF I^{131} DISAPPEARANCE

Per cent fall in half-life was obtained by comparing the half-life before or after salicylate administration with that during treatment. When both pre- and postsalicylate determinations were available (Table IIIc), they were averaged and the result compared to the value obtained during salicylate administration. The regression line is the best fit line through these points by the method of least squares. The coefficient of correlation (r) is 0.60 ($p < 0.01$).

TABLE IV

The effect of salicylate therapy on the concentration of thyroxine binding sites of serum thyroxine-binding protein

Patient	Before salicylate therapy			During salicylate therapy*			After salicylate therapy		
	PBI	TBP† sites	Free T_4‡	PBI	TBP sites	Free T_4	PBI	TBP sites	Free T_4
	μg. %			μg. %			μg. %		
R. P.				3.4	2.1×10^{-7} M	4.6×10^{-11} M	4.4	2.1×10^{-7}	6.6×10^{-11} M
T. K.				4.4	1.9	5.5	7.2	2.3	8.9
K. C.				3.8	3.1	3.5	6.6	2.8	8.4
W. C.	5.3	2.8×10^{-7} M	6.0×10^{-11} M	3.4	2.8	3.2	4.5	2.5	4.7
F. K.				3.1	2.3	3.7	5.4	2.4	7.3
J. L.				3.0	2.0	4.1	4.8	2.1	6.7
Mean				3.5	2.4	4.1	5.5	2.4	7.1

* Thyroxine binding was studied after four to eight weeks of salicylate therapy and again four to six weeks after discontinuation of salicylate treatment.

† TBP refers to thyroxine-binding protein.

‡ T_4 refers to free thyroxine calculated according to Robbins and Rall (38).

i.e., the EOI pool was not known because the PBI was falling and the distribution space was not determined.

Thyroxine binding

The major portion of thyroxine is normally bound in the plasma to a specific thyroxine binding alpha-globulin (TBP) (38). A decrease in the amount of this protein, a change in the affinity constant for thyroxine, or competition by salicylate or one of its conjugates for the binding sites would reduce the per cent of bound thyroxine and possibly change thyroxine metabolism. Salicylate administration was not associated with any significant alteration in the concentration of TBP sites (Table IV). Hence, free thyroxine falls concomitantly with the decrease in PBI. Furthermore, there was no alteration in the distribution of thyroxine among the serum proteins when only small quantities of thyroxine were used, suggesting that, with physiologic thyroxine concentrations, there was no effective competition for binding sites between salicylate and thyroxine. Thus, a change in the binding of radiothyroxine is not the explanation for the increased fractional rate of disappearance of radiothyroxine. The present situation appears to be an exception to the dependence of the degradation rate upon the level of free thyroxine as postulated by Robbins and Rall (38).

TABLE V

Effect of salicylates on thyroid uptake of I^{131}

Patient	Per cent of administered dose in thyroid								
	Control period			Salicylate period			Postcontrol period		
	2 hr.	5 hr.	24 hr.	2 hr.	5 hr.	24 hr.	2 hr.	5 hr.	24 hr.
K. C.	17	23	38	3	7	19	9	13	31
C. C.	13	25	50	8	17	26	15	21	43
W. C.	10	16	26	6	9	17	8	13	20
J. B.	17	23	34	5	8	15	10	13	25
R. B.	12	17	30	5	8	15			
F. K.	13	20	29	7	12	21	10	14	24
C. M.	6	11	18	3	5	9	6	8	14
J. L.	5	10	16	3	4	6	4	9	16
R. Pi.	10	17	33	11	17	27			
Mean	11	18	30	6	10	17	9	13	25
Standard deviation	4	5	10	2	4	7	3	4	9

Radioiodine studies

The possibility that the reduction in PBI was in part due to a depression in thyroid function and ultimately in hormone synthesis was explored in several ways. The 2, 5 and 24-hour thyroid radioiodine uptake was observed in nine patients before and during salicylate therapy, and in seven of these patients six weeks after discontinuing the drug (Table V). The mean fall in uptake was 45 per cent of the control value at 2 hours, 44 per cent at 5 hours, and 43 per cent at 24 hours; in comparison to the postsalicylate values the reduction in uptake was less, but still 33, 23 and 36 per cent at 2, 5 and 24 hours, respectively. Serial determinations were not performed but the reduction recorded in Table V was observed after two weeks in Patient C. M., after four weeks in Patients K. C. and J. L., and after eight weeks in the remainder.

The kidney is the chief organ competing with the thyroid gland for circulating iodide. Thus, an increase in the renal clearance of I^{131} could produce an apparent depression of thyroid uptake. That this was not the case is shown in Figure 4. No change in the renal clearance of iodide occurred during salicylate administration, the mean clearance being 53 ml. per minute both on and off salicylate therapy. This value is slightly higher than that usually observed (39–41). Bricker and Hlad (41) have demonstrated that a water diuresis up to 6 ml. per minute does not alter renal clearance of radioiodine, but they did not carry the diuresis to the rates of urine flow used in the present study.

On the other hand, thyroid clearance of radioiodide was reduced in each patient during chronic salicylate administration (Figure 4). This indicates that the diminution in radioiodine uptake (Table V) is indeed an expression of depressed thyroid function. The control thyroid clearance and the radioiodine distribution space before and during salicylate therapy agree with the values reported by Berson, Yalow, Sorrentino, and Roswit (40).

The reduction in thyroid uptake and clearance of radioiodine during chronic salicylate administration could result either from inhibition of the iodide concentrating mechanism or from interference with the utilization of the accumulated iodide for hormone synthesis. The former possibility is conveniently measured in rats as the concentration gradient of iodide in the thyroid to that in the serum or plasma (T/S ratio) when hormone synthesis is blocked. Even at high serum concentrations acute salicylate administration does not depress the ability of the rat thyroid to concentrate iodide (Table VI). In contrast, sodium thiocyanate produced the expected marked depression of the T/S ratio.

Para-hydroxy benzoic acid may interfere with thyroidal organification of iodide (42). Thus,

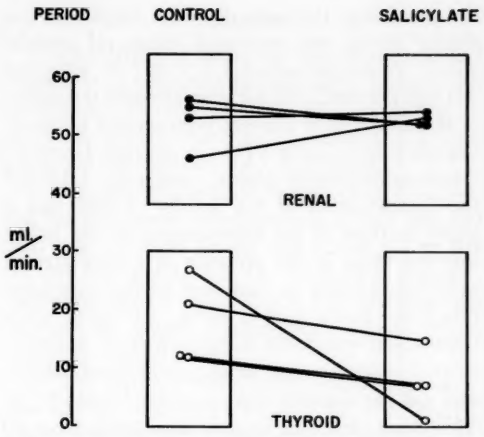

FIG. 4. EFFECT OF SALICYLATE ADMINISTRATION ON I^{131} CLEARANCE

The clearance determinations were done after six weeks of salicylate administration; in three subjects the control value was obtained before salicylate treatment, and in the other the control study was done four weeks after discontinuation of salicylates. Each point represents the mean of six or more clearance values obtained at half-hour intervals.

TABLE VI

Effect of salicylate and thiocyanate on the thyroid/serum iodide ratio in rats

Treatment	Mean blood level	Mean T/S ratio
	mg.%	
Control (15)*		147 ± 10†
Salicylate (15)	51 (15)*	137 ± 8
Na SCN (12)		2.7 ± 0.3

* Figures in parentheses refer to the number of rats used.
† Mean ± standard error. The "t" test reveals that the salicylate treated rats do not differ significantly from the control ($0.5 > p > 0.4$).

the possibility that salicylic acid might have a similar action was evaluated using rat thyroid slices. A concentration of 3×10^{-3} M salicylate (41 mg. per cent) did not interfere with the ability of thyroid slices to convert incorporated iodide to organic forms (Table VII); in contrast, 1-methyl-2-mercaptoimidazole almost completely inhibited the organification of the iodide. There was a slight decrease in the incorporation of the iodide into the slices in the presence of a concentrated salicylate solution as compared to the incorporation in the Krebs-Ringer media. However, when comparison was made using sera from salicylate treated patients as the incubation medium, salicylates did not diminish incorporation (Table VII). This is not surprising since at these levels about 70 per cent of the salicylate is bound to plasma protein (43–45). The possibility of thyroid inhibition by some salicylate derivative is thus also excluded. Furthermore, the addition of known amounts of buffered sodium salicylate in the concentration obtained clinically in this study had no direct effect on the uptake and organification of radioiodine by rat thyroid slices (Figure 5).

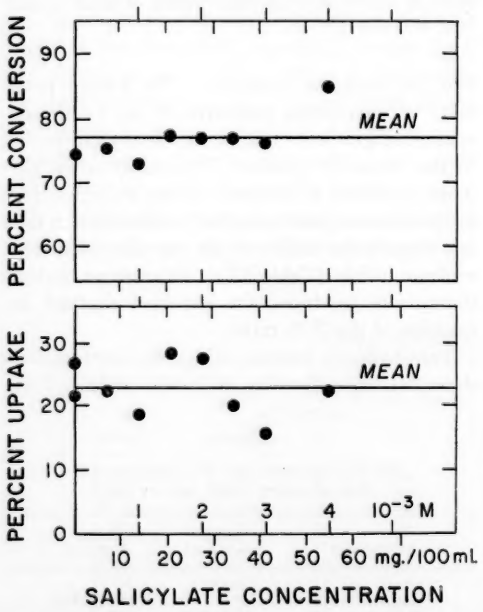

FIG. 5. EFFECT OF SALICYLATE ON THE UPTAKE AND ORGANIFICATION OF I^{131} BY RAT THYROID SLICES

Rat thyroid tissue was incubated in human serum to which varying concentrations of buffered sodium salicylate were added.

COMMENT

An increase in the basal metabolic rate associated with a negative nitrogen balance and weight loss on a fixed caloric intake can be produced in euthyroid patients by salicylate administration. In patients with myxedema the serum cholesterol is also lowered by salicylate treatment. On the other hand, the clinical findings generally regarded as characteristic of hyperthyroid hypermetabolism, such as heat intolerance, hyperkinesis, emotional lability, and tachycardia, are lacking.

Concomitant with the increase in oxygen consumption, salicylate administration produces a prompt reduction in the serum protein bound iodine concentration. The abrupt decrease in the rate of release of thyroidal I^{131} with salicylate administration (46) probably contributes significantly to the rapid fall in the PBI. On the other hand, the depression in the thyroidal uptake and clearance of I^{131} probably does not play an important role in the early reduction of the PBI because of the large stores of stable iodine in the normal gland. Whatever the contributing causes, a new steady state is attained within the first 10

TABLE VII

The effect of salicylate on the uptake and conversion of I^{131} by rat thyroid slices *

Experiment	Incubation medium	Per cent of total I^{131} in slices	Per cent of slice I^{131} as organic I^{131}
I	Krebs-Ringer phosphate	62	86
	1×10^{-3} M sodium salicylate	54	70
	3×10^{-3} M sodium salicylate	47	78
	1×10^{-3} M 1-methyl-2-mercaptoimidazole	40	1.0
II	Normal human serum	48	85
	Salicylate serum (41 mg. % or 3×10^{-3} M)	44	75

* Forty mg. of slices from thyroids of rats kept on a low iodine diet for 26 days, incubated at 37° C. for three hours in 3.0 ml. of Krebs-Ringer phosphate medium, pH 7.4, containing the agents indicated. In Experiment II serum from patients before and during salicylate therapy was used in place of the Krebs-Ringer phosphate medium.

days of salicylate administration, and the PBI is not lowered to frankly hypothyroid levels.

In view of the finding of a normal degradation rate together with depressed thyroid function during chronic salicylate treatment, the failure of the PBI to continue to fall is at present not readily explained. This disparity could result from a methodological artifact in the estimation of the degradation rate, for it can be shown (47) that under certain circumstances in open systems calculation of pool size by the intercept method contains an error that is proportional to the slope. Furthermore, Ingbar and Freinkel (37) noted a discrepancy between the amount of thyroid hormone synthesized and degraded, calculated by independent means, in patients with hypermetabolism due to thyrotoxicosis. Sterling and Chodos (36) observed an increased fractional rate of disappearance of radiothyroxine, a normal PBI, an enlarged EOI pool, and an increased degradation rate in patients with hypermetabolism of malignancy and infection. If the augmented degradation rate continued for a period of time, the thyroidal stores of I^{127} would become depleted and an increase in the thyroidal uptake of I^{131} might be expected. This, however, was not observed in the patients with leukemia and other causes of extrathyroidal hypermetabolism studied by Meckstroth, Rapport, Curtis, and Simcox (48) and by Skanse (49). Thus, extrathyroidal hypermetabolism due to malignancy and infection may be another example of a disparity between peripheral thyroxine metabolism, measured by the intercept method, and the level of function of the thyroid gland, as measured by thyroidal uptake, clearance, or release rate.

The failure of *acute* salicylate administration to reduce the T/S ratio in the rat or to interfere with the incorporation and organification of I^{131} by rat thyroid slices suggests that thyroid inhibition is not the result of a direct effect of salicylate on the thyroid gland. An indirect action, presumably via the pituitary or higher centers, similar to that of thyroxine is suggested. The localization of this indirect inhibition and the implication of a decrease in available thyrotropin form the subject of the following report (46).

SUMMARY

1. The effect of salicylate administration was studied in 24 euthyroid patients and 1 patient with myxedema; 19 received 6.0 to 8.1 Gm. of sodium salicylate per day for 8 to 15 weeks and 6 were given 9.0 Gm. per day for 7 to 10 days.

2. Oxygen consumption during chronic salicylate therapy (mean concentration, 37 mg. per 100 ml.) was increased 33 per cent (from 221 to 294 ml. per min. per 1.73 $M.^2$). There was a significant correlation ($r = 0.72$, $p < 0.01$) between the increase in oxygen consumption and the serum salicylate concentration.

3. The serum protein bound iodine concentration was reduced from a mean of 5.5 to 3.8 micrograms per 100 ml. during chronic salicylate administration and was maintained at this level until the salicylate was discontinued.

4. The fractional rate of disappearance of radiothyroxine from the circulation rose from a mean of 11.3 per cent per day to 15.1 per cent per day during chronic salicylate therapy and returned to 11.0 per cent per day with cessation of salicylate treatment. There was a significant correlation ($r = 0.60$, $p < 0.01$) between the increase in the fractional rate of radiothyroxine disappearance and the rise in oxygen consumption.

5. The mean reduction in 2, 5 and 24 hour uptake of radioiodine during chronic salicylate treatment was 45 per cent of the control value at 2 hours, 44 per cent at 5 hours, and 43 per cent at 24 hours.

6. Thyroidal clearance of radioiodine was depressed in four patients during chronic salicylate administration whereas renal clearance was not significantly altered.

7. Acute salicylate administration in rats did not reduce the thyroid/plasma ratio of radioiodine.

8. The incubation of rat thyroid slices in serum of salicylate-treated patients did not interfere with the incorporation or organification of radioiodine.

9. These findings suggest that an important action of salicylate administration is a depression of thyroid function, presumably via the pituitary or higher centers.

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REFERENCES

1. Denis, W., and Means, J. H. The influence of salicylate on metabolism in man. J. Pharmacol. exp. Ther. 1916, **8**, 273.
2. Cochran, J. B. The respiratory effects of salicylate. Brit. med. J. 1952, **2**, 964.
3. Cochran, J. B. Further observations on the metabolic stimulating effect of salicylate. Brit. med. J. 1954, **1**, 733.
4. Meade, B. W. The effect of certain hydroxybenzoic acids on the oxygen consumption of Wistar rats. Ann. rheum. Dis. 1954, **13**, 60.
5. Tenney, S. M., and Miller, R. M. The respiratory and circulatory actions of salicylate. Amer. J. Med. 1955, **19**, 498.
6. Reid, J. A new outlook on the action of salicylate. Scot. med. J. 1957, **2**, 91.
7. Brody, T. M. The action of sodium salicylate and related compounds on tissue metabolism *in vitro*. J. Pharmacol. exp. Ther. 1956, **117**, 39.
8. Sproull, D. H. A peripheral action of sodium salicylate. Brit. J. Pharmacol. 1954, **9**, 262.
9. Smith, M. J. H., and Jeffrey, S. W. The effect of salicylate on oxygen consumption and carbohydrate metabolism in the isolated rat diaphragm. Biochem. J. 1956, **63**, 524.
10. Cutting, W. C., Rytand, D. A., and Tainter, M. L. Relationship between blood cholesterol and increased metabolism from dinitrophenol and thyroid. J. clin. Invest. 1934, **13**, 547.
11. Alwall, N., and Scheff-Pfeifer, I. Über den Synergismus Dinitrophenol-Thyroxin und Methylen blau-thyroxin am künstlich durchströmten isolierten Hundebein. Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak. 1936, **184**, 296.
12. Simkins, S. Dinitrophenol and desiccated thyroid in the treatment of obesity. J. Amer. med. Ass. 1937, **108**, 2110 and 2193.
13. Goldberg, R. C., Wolff, J., and Greep, R. O. Studies on the nature of the thyroid-pituitary interrelationship. Endocrinology 1957, **60**, 38.
14. Lardy, H. A., and Wellman, H. Oxidative phosphorylations: Role of inorganic phosphate and acceptor systems in control of metabolic rates. J. biol. Chem. 1952, **195**, 215.
15. Packer, L. Coupled phosphorylation in rat heart muscle sarcosomes. Arch. Biochem. 1957, **70**, 290.
16. Smith, M. J. H., Meade, B. W., and Bornstein, J. The effect of salicylate on glycosuria, blood glucose, and liver glycogen of the alloxan-diabetic rat. Biochem. J. 1952, **51**, 18.
17. Wolff, J., Rubin, L., and Chaikoff, I. L. The influence of 2,4-dinitrophenol on plasma protein-bound iodine. J. Pharmacol. exp. Ther. 1950, **98**, 45.
18. Goldberg, R. C., Wolff, J., and Greep, R. O. The mechanism of depression of plasma protein bound iodine by 2,4-dinitrophenol. Endocrinology 1955, **56**, 560.
19. Castor, C. W., and Beierwaltes, W. The effect of 2,4-dinitrophenol on thyroid function in man. J. clin. Endocr. 1956, **16**, 1026.
20. Austen, F. K., Rubini, M. E., Meroney, W. H., and Wolff, J. Salicylates and thyroid function (abstract). J. clin. Invest. 1957, **36**, 870.
21. Keller, W. J., Jr. A rapid method for the determination of salicylates in serum or plasma. Amer. J. clin. Path. 1947, **17**, 415.
22. Peters, J. H. The determination of creatinine and creatine in blood and urine with the photoelectric colorimeter. J. biol. Chem. 1942, **146**, 179.
23. Smith, P. K. Certain aspects of the pharmacology of the salicylates. Pharmacol. Rev. 1949, **1**, 353.
24. Smith, P. K. Review of pharmacology and metabolism of aspirin and salicylic acid *in* Aspirin—Recent Advances in its Pharmacology and Clinical Use. St. Louis, Monsanto Chemical Company, 1956, p. 1.
25. Schoenheimer, R., and Sperry, W. M. A micromethod for the determination of free and combined cholesterol. J. biol. Chem. 1934, **106**, 745.
26. Pearson, S., Stern, S., and McGavack, T. H. A rapid, accurate method for the determination of total cholesterol in serum. Analyt. Chem. 1953, **25**, 813.
27. Barker, S. B., Humphrey, M. J., and Soley, M. H. The clinical determination of protein-bound iodine. J. clin. Invest. 1951, **30**, 55.
28. Sterling, K., Lashof, J. C., and Man, E. B. Disappearance from serum of I^{131}-labeled l-thyroxine and l-triiodothyronine in euthyroid subjects. J. clin. Invest. 1954, **33**, 1031.
29. Robbins, J. Reverse-flow zone electrophoresis. A method for determining the thyroxine-binding capacity of serum protein. Arch. Biochem. 1956, **63**, 461.
30. Keating, F. R., Jr., Wang, J. C., Luellen, T. J., Williams, M. M. D., Power, M. H., and McConahey, W. M. The measurement of the iodine-accumulating function of the human thyroid gland. J. clin. Invest. 1949, **28**, 217.
31. Morton, M. E., and Chaikoff, I. L. The formation *in vitro* of thyroxine and diiodotyrosine by thyroid tissue with radioactive iodine as indicator. J. biol. Chem. 1943, **147**, 1.
32. Alexander, W. D., and Johnson, K. W. M. Comparison of the metabolic stimulating action of aspirin and thyroid. Nature (Lond.) 1956, **178**, 259.
33. Austen, F. K., Rubini, M. E., and Meroney, W. H.

The effect of salicylate administration on nitrogen, calcium, and phosphorus balance. In preparation.

34. Alexander, W. D., and Johnson, K. W. A comparison of the effects of acetylsalicylic acid and DL-triiodothyronine in patients with myxoedema. Clin. Sci. 1956, **15**, 593.
35. Stamler, J., Silber, E. N., Miller, A. J., Akman, L., Bolene, C., and Katz, L. N. The effect of thyroid- and of dinitrophenol-induced hypermetabolism on plasma and tissue lipids and atherosclerosis in the cholesterol-fed chick. J. Lab. clin. Med. 1950, **35**, 351.
36. Sterling, K., and Chodos, R. B. Radiothyroxine turnover studies in myxedema, thyrotoxicosis, and hypermetabolism without endocrine disease. J. clin. Invest. 1956, **35**, 806.
37. Ingbar, S. H., and Freinkel, N. Simultaneous estimation of rates of thyroxine degradation and thyroid hormone synthesis. J. clin. Invest. 1955, **34**, 808.
38. Robbins, J., and Rall, J. E. The interaction of thyroid hormones and protein in biological fluids. Recent Progr. Hormone Res. 1957, **13**, 161.
39. Myant, N. B., Pochin, E. E., and Goldie, E. A. G. The plasma iodide clearance rate of the human thyroid. Clin. Sci. 1949, **8**, 109.
40. Berson, S. A., Yalow, R. S., Sorrentino, J., and Roswit, B. The determination of thyroidal and renal plasma I^{131} clearance rates as a routine diagnostic test of thyroid dysfunction. J. clin. Invest. 1952, **31**, 141.
41. Bricker, N. S., and Hlad, C. J., Jr. Observations on the mechanism of the renal clearance of I^{131}. J. clin. Invest. 1955, **34**, 1057.
42. Taurog, A., Chaikoff, I. L., and Franklin, A. L. The structural specificity of sulfanilamide-like compounds as inhibitors of the *in vitro* conversion of inorganic iodide to thyroxine and diiodotyrosine by thyroid tissue. J. biol. Chem. 1945, **161**, 537.
43. Smith, P. K., Gleason, H. L., Stoll, C. G., and Ogorzalek, S. Studies on the pharmacology of salicylates. J. Pharmacol. exp. Ther. 1946, **87**, 237.
44. Lester, D., Lolli, G., and Greenberg, L. A. The fate of acetylsalicylic acid. J. Pharmacol. exp. Ther. 1946, **87**, 329.
45. Seeberg, V. P., Hansen, D., and Whitney, B. Absorption and distribution of salicylamide. J. Pharmacol. exp. Ther. 1951, **101**, 275.
46. Wolff, J., and Austen, F. K. Salicylates and thyroid function. II. The effect on the thyroid-pituitary interrelation. J. clin. Invest. 1958, **37**, 1144.
47. Lewallen, C. G., and Berman, M. Personal communication.
48. Meckstroth, C. V., Rapport, R. L., Curtis, G. M., and Simcox, S. J. The laboratory diagnosis of extrathyroidal hypermetabolism. J. clin. Endocr. 1952, **12**, 1373.
49. Skanse, B. Radioactive iodine in the diagnosis of thyroid disease. Acta med. scand. 1949, Suppl. **235**.

SALICYLATES AND THYROID FUNCTION. II. THE EFFECT ON THE THYROID–PITUITARY INTERRELATION

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In the preceding paper (1) it was pointed out that the administration of sodium salicylate to man induces a number of changes suggestive of thyrotoxicosis. There is an elevated basal metabolic rate (BMR), negative nitrogen balance, a fall in the cholesterol level of the hypothyroid subject, and an accelerated fractional disappearance rate of labeled thyroxine from the circulation. Paradoxically, salicylate-induced hypermetabolism is not accompanied by certain of the clinical characteristics of hyperthyroidism and is associated with depressed thyroid function as measured by thyroidal radioiodine uptake and clearance, or the serum protein bound iodine (PBI) concentration. The salicylate level in the serum of such patients is not high enough to interfere with *in vitro* iodine metabolism in rat thyroid slices, and in *acute* experiments in rats no depression of the iodide concentrating mechanism can be demonstrated at blood salicylate levels comparable to those attained in the above patients (1). Similar findings had previously been recorded in rats and in man treated with 2,4-dinitrophenol (2–4). It was subsequently shown (5) that this thyroid depression produced by 2,4-dinitrophenol probably resulted from inhibition at a pituitary or higher level.

The absence of a direct or immediate effect of salicylates on thyroid tissue, despite marked thyroid depression upon more prolonged exposure to the drug, suggested that this effect may also result from a decrease in thyroid stimulation by thyrotropin (TSH). It is the purpose of this report to suggest a locus of salicylate action and to present some observations on the nature of the inhibitory effect.

MATERIALS AND METHODS

Three euthyroid patients were studied to evaluate the effect of salicylates on the rate of release of radioiodine from the thyroid gland. The technique was similar to that described by Goldsmith, Stanbury, and Brownell (6). Each patient, while fasting, was given approximately 125 microcuries of radioiodine, and 48 hours later was started on 1-methyl-2-mercaptoimidazole, 30 mg. every six hours, to block the reaccumulation of radioiodine. Counts over the thyroid region were measured each morning with a wide angle scintillation counter. Body background was taken as the thigh count, and the logarithm of the counts remaining in the thyroid after correction for body background and physical decay was plotted against time to determine half-life and rate of release of thyroidal I^{131}.

Thyrotropin [1] (TSH), dissolved in physiologic saline and injected intramuscularly, was given during salicylate administration to seven patients in a dose of 5 U.S.P. units every 12 hours. In five patients thyrotropin was started immediately after completion of a 2, 5, and 24 hour uptake of I^{131}. Values were corrected for residual counts remaining from any previous experiment. Two other patients received thyrotropin by the same dose schedule while the thyroidal release rate was being studied.

Release of I^{131} from the propylthiouracil-blocked rat thyroid was measured according to the method of Wolff (7) with minor modifications. A rate meter operating off the same detector was used to center the thyroid area prior to counting. The diet used was a modified Remington diet; because rats kept on this diet for prolonged periods showed biological half-lives of thyroidal I^{131} of well under 24 hours, rats were fed this diet for only 12 to 16 days prior to the release studies. Each animal served as its own control and the pooled values of each group were plotted semilogarithmically to obtain half-lives of thyroidal I^{131}.

Sodium salicylate, salicylamide, 2,5-, 2,6-, 3,5-dihydroxybenzoic acid, and *m*- and *p*-hydroxybenzoic acid were obtained commercially. Salicyluric acid was synthesized by the method of Bondi (8), M.P. uncorrected equals 166° C. Before injection the acids were neutralized to pH 6.0; higher pH values were avoided to prevent air oxidation of the phenols. Total plasma "salicyl" (salicylate and salicylurate) and 2,6-dihydroxybenzoic acid were determined by a Fe^{+++} ion method (9), at 530 mμ and 560 mμ, respectively. Gentisic acid was determined after acidification of the serum or plasma to pH 5.5 with 0.1 M acetate buffer either by a modified Fe^{+++} ion method or with the phenol reagent (10, 11).

Goiter prevention experiments were carried out as

[1] Supplied by the Armour Laboratories, Chicago, Ill.

TABLE I

Response of thyroid uptake and serum protein bound iodine to exogenous thyrotropin during chronic salicylate administration

Patient	Salicylate control				Thyrotropin administration			
	Radioiodine uptake (*per cent*)				Radioiodine uptake (*per cent*)			
	2 hr.	5 hr.	24 hr.	PBI	2 hr.	5 hr.	24 hr.	PBI
				μg. %				μg. %
K. C.	3	7	19	4.1	33	43	66	7.3
R. P.	5	10	26	3.7	24	40	66	7.1
C. C.	8	17	26	3.3	12	36	64	8.6
R. B.*	5	8	15	5.0	11	20	46	8.6
J. L.*	3	4	6	3.6	9	17	27	6.3
Mean	5	9	18	3.9	18	31	54	7.6

* Thyrotropin stimulation (10 U per day) for one day only, whereas given on three successive days to the other patients.

previously described (5). High dietary levels of sodium salicylate were required to yield measureable effects but these could not be given from the start of the experiment because they led to considerable weight loss. On the other hand, when the sodium salicylate in the diet (Purina Lab Chow) was gradually increased 0.3 per cent per week until a level of 1.2 per cent was reached, no weight loss occurred. The rats were then divided into two groups, one receiving 0.1 per cent propylthiouracil together with the 1.2 per cent sodium salicylate in the diet, the other group, 1.2 per cent sodium salicylate only. Two other groups of rats served as controls—one untreated, the other receiving 0.1 per cent propylthiouracil in the diet. After 11 days on this regimen the thyroids were weighed and prepared for routine histological examination, and the thyroid to plasma (T/S) iodide ratio was determined as described previously (4).

Radioactive salicylic acid carboxyl C^{14} of specific activity, 1 μc. per μM, was used to investigate localization of the drug. The radioactivity was 97 per cent salicylate as judged by ascending paper chromatography in *n*-butanol equilibrated with an equal volume of 2 N NH_4OH (Rf equals 0.51 to 0.56), in *n*-butanol-dioxane (80:20) equilibrated with an equal volume of 2N NH_4OH (Rf equals 0.60 to 0.64), in benzene-acetic acid-H_2O (2:2:1), or in *n*-butanol-acetic acid-H_2O (4:1:1) (12). The remaining radioactivity consisted of two separate bands, both of low Rf value. Chromatograms were scanned with a recording rate meter and also exposed to no-screen X-ray film. The specific activity was sufficiently high so that small samples of tissue could be counted directly, without extraction or ashing. Two and five-tenths to 7.0 mg. of salicylate were injected intraperitoneally into male rats (100 to 150 Gm.) which were sacrificed and exsanguinated at intervals thereafter. Tissues were removed, ground with water in an all glass homogenizer, and an equivalent of 2 to 10 mg. of tissue (wet weight) was mounted on copper planchets and counted. Appropriate self absorption corrections were made.

RESULTS AND DISCUSSION

The responsiveness of the salicylate depressed thyroid gland to exogenous thyrotropin is illustrated in Table I. Both the PBI level and I^{131} uptake showed striking elevations following TSH stimulation in five patients maintained on chronic salicylate therapy.

The administration of sodium salicylate markedly slowed the rate of release of thyroidal I^{131} (Figures 1 and 2), increasing the half-life from 10.7 and 12.4 days, respectively, to one too great for accurate definition. The reduction in release rate was apparent within 36 hours after beginning salicylate; on discontinuation of the drug the slope once again approximated that obtained prior to salicylate treatment (Figure 1). Since it has been demonstrated that one of the main actions of thyrotropin is to stimulate release of hormone from the gland (7), these results suggest that salicylates may in some manner interfere with the action of thyrotropin. That this interference is not due to an inability of the salicylate-depressed gland to respond to thyrotropin has already been noted in Table I. Inhibition of release of thyroidal I^{131} by salicylates (Figure 2) (serum concentration, 35 to 51 mg. per 100 ml.) is also readily overcome by exogenous thyrotropic stimulation despite continuation of salicylate administration. Furthermore, the acceleration of release of thyroidal I^{131}, so characteristic of thyrotropic action (7), is not interrupted by salicylate administration sufficient to produce a blood level of 28 to 42 mg. per 100

ml. (Figure 3). Since the amount of TSH was greater than the minimal effective dose, it is still possible that a small decrease in sensitivity to TSH resulted from salicylate treatment but could not be demonstrated with the present method.

If the thyrotropin available to the thyroid is diminished by salicylates, this drug might be expected to interfere with goiter formation in propylthiouracil-treated rats. That this did occur is shown in Table II. Whereas propylthiouracil-treated rats showed thyroid gland enlargement to a mean weight of 26 mg. per 100 Gm. of body weight, the concomitant administration of sodium salicylate held the final mean thyroid weight to 13 mg. per 100 Gm. of body weight ($p \ll 0.001$). Goiter prevention was not complete, however, since untreated and salicylate-treated controls had mean thyroid weights of approximately 7 mg. per 100 Gm. body weight ($p < 0.001$). Histological examination of the glands of some of the rats receiving both drugs simultaneously showed increased cell height and a moderate depletion of the colloid in the central follicles. Other glands closely resembled the controls. This variability in the histological picture is reflected in the relatively large standard deviation of the thyroid weights of this group. In contrast, thyroids of propylthiouracil-treated rats showed the usual picture of complete absence of colloid and very tall collumnar epithelium with marked papillary infolding. Rats treated with salicylate and propylthiouracil tended to eat more of the diet than rats kept on propylthiouracil alone. Thus, the intake of goitrogen in the doubly treated group was at least that of the propylthiouracil controls. It is, however, still possible that the rate of metabolism of propylthiouracil was sufficiently increased by salicylate to lead to diminished goitrogenicity. Salicylate treatment alone led to minimal flattening of the epithelium and some pyknosis of the nuclei. The T/S ratio of the salicylate and propylthiouracil-treated rats showed little change from the propylthiouracil group ($0.1 < p < 0.2$). Results of the studies on the T/S ratio and the histology closely resemble those of the incomplete goiter prevention observed with 2,4-dinitrophenol (5). It is probable that the discrepancy between thyroid weight and the T/S ratio is further evidence that suppression of the TSH level is only partial, since the T/S ratio is known to be more sensitive to slight thyrotropic stimulation (13).

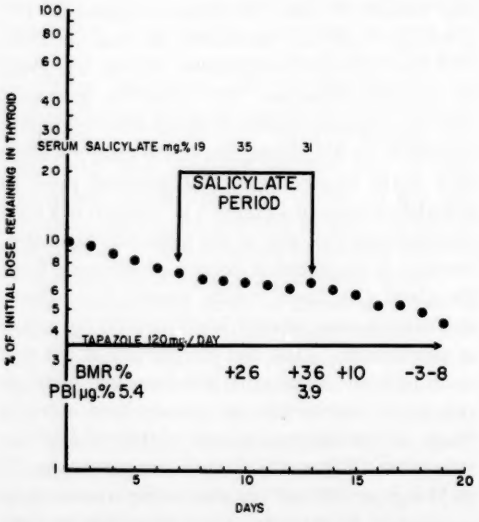

FIG. 1. THE EFFECT OF SALICYLATE ADMINISTRATION ON THE RELEASE OF I^{131} FROM THE HUMAN THYROID GLAND

1-methyl-2-mercaptoimidazole was started 48 hours after an oral dose of 125 µc. of I^{131}. Seven and two-tenths Gm. of sodium salicylate per day was given during the salicylate period.

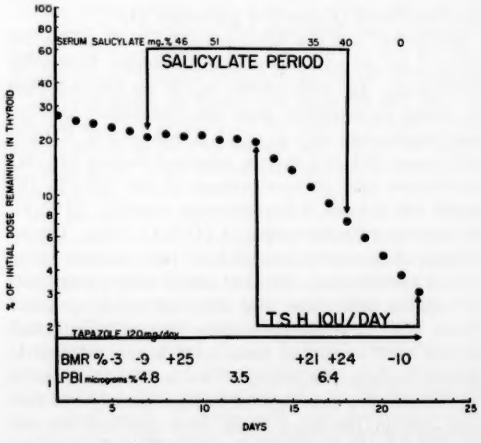
% OF INITIAL DOSE REMAINING IN THYROID
SERUM SALICYLATE mg.% 46 51 35 40 0
SALICYLATE PERIOD
T.S.H. 10U/DAY
TAPAZOLE 120mg/day
BMR % -3 -9 +25 +21 +24 -10
PBI micrograms % 4.8 3.5 6.4
DAYS

FIG. 2. THE EFFECT OF SALICYLATE ADMINISTRATION ON THE RELEASE OF I^{131} FROM THE HUMAN THYROID GLAND

Conditions as for Figure 1. Five U.S.P. units of thyrotropin was injected intramuscularly every twelve hours.

TABLE II

Goiter prevention in rats by salicylate

Treatment	Mean initial weight	Mean final weight	Mean thyroid weight	Mean T/S ratio
	Gm.	*Gm.*	*mg./100 Gm.*	
Control (10)*	121†	170	6.6 ± 0.66‡	37 ± 13‡
0.1% propylthiouracil (10)	118†	142	26.2 ± 6.7	190 ± 47
0.1% propylthiouracil + 1.2% salicylate (10)	141	140	12.7 ± 3.9	142 ± 70
1.2% salicylate (8)	146	156	6.8 ± 0.82	32 ± 14

* Numbers in parentheses indicate number of rats.

† Because of pretreatment of the salicylate groups, these animals were chosen so that the final weights would be approximately equal.

‡ Standard deviation of the mean.

Diminished thyrotropic response seemed thus to be implicated, probably at a pituitary or higher level, and we were therefore interested in the report by Pallot and Eberhardt (14) that the C^{14} of carboxyl-labeled salicylate seemed to be concentrated in the adenohypophysis (tissue/serum ratio equals 0.7 for anterior pituitary, and 0.09 for the other organs). We were able to confirm a "relative" concentration of radioactivity in the anterior pituitary gland when compared to brain, but other organs showed a similar degree of concentration (Table III). Chromatographic analysis revealed that more than 90 to 95 per cent of the radioactivity of the tissues listed in Table III behaved like salicylate one hour after injection of labeled salicylate. In most tissues, including the anterior pituitary gland but not the brain or thyroid gland, traces of salicylglucuronide and salicyl-

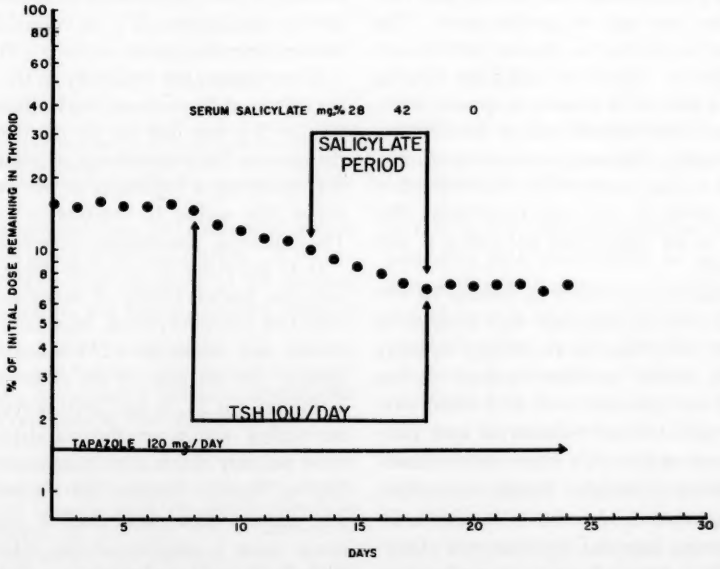

FIG. 3. THE EFFECT OF SALICYLATE ADMINISTRATION ON THE RELEASE OF I^{131} FROM THE HUMAN THYROID GLAND

Conditions as in Figure 2.

TABLE III

Distribution of salicylate C^{14} in rat tissues*

	Ratio of tissue C^{14} to plasma C^{14}			
Interval after injection (hrs.)	0.5	1.0	2.0	4.0
No. of rats	2	5	2	2
Liver	0.26	0.39	0.28	0.30
Kidney cortex	0.53	0.53	0.67	0.54
Adrenal (whole gland)	0.18	0.31	0.24	0.25
Spleen	0.15	0.23	0.15	0.15
Testis	0.14	0.18	0.13	0.16
Muscle (gastrocnemius)	0.11	0.19	0.12	0.10
Heart (left ventricle)	0.28	0.36	0.26	0.37
Lung		0.37	0.31	0.25
Thyroid gland	0.23	0.43	0.25	0.27
Submaxillary gland	0.21	0.28	0.21	0.22
Cerebral cortex	0.06	0.12	0.06	0.07
"Hypothalamus"†	0.11	0.10	0.10	0.07
Anterior pituitary	0.13	0.29	0.30	0.24
Posterior pituitary	0.07	0.07	0.09	0.16
Brain stem	0.04	0.12	0.06	0.07
Cerebellum	0.04	0.11	0.07	0.07

* Specific activity of 1 μc. per μM; 7 to 24 μc. injected per rat intraperitoneally.

† Includes tuber cinereum and floor of third ventricle.

urate could be demonstrated on films exposed to chromatograms for two to four months. Too little activity was present to be detected by the scanner. In the liver, kidney and plasma, two to six per cent of the radioactivity behaved chromatographically as glucuronides, and in liver and kidney one to two per cent as gentisic acid. The latter could not be checked in alkaline solvents because of oxidation. Since the salicylate concentration is below that of the plasma, it appears likely that the effect is not specific and is possibly related to vascularity. It must be remembered, furthermore, that a drug need not be concentrated in the organ in which it acts, and conversely, that concentration in an organ does not prove it acts there specifically.

A comparison of the effect of release of I^{131} from the rat thyroid by salicylate with some of its congeners was undertaken in an attempt to study the mechanism of the inhibitory action. It has been reported that gentisic acid (2,5-dihydroxybenzoic acid) (15, 16) and γ-resorcylic acid (2,6-dihydroxybenzoic acid) (17) have antirheumatic activity, the latter in smaller dosage than either salicylate or gentisate. As shown in Table IV both of these drugs depress the release rate of thyroidal I^{131}. On a dosage basis, γ-resorcylate was less active than salicylate; however, strict comparison is not possible, since blood levels comparable to those obtained with sodium salicylate (about 50 mg. per 100 ml.) could never be attained with the diphenolic carboxylic acids, even when these were given every four hours and at three times the daily dose. Maximum blood levels attained were 2 to 11 mg. per 100 ml. for gentisate and 4 to 18 mg. per 100 ml. for γ-resorcylate (measured at 4 hours, *i.e.*, just prior to the next injection). Similar difficulties in maintaining blood levels with gentisic acid have been encountered clinically (15, 16) but lower levels apparently suffice to obtain adequate therapeutic response.

COMMENTS

Thyroxine, dinitrophenol (DNP), and salicylate evoke many similar responses in man or the experimental animal (Table V). In addition to the similarities noted above, both salicylate and thyroxine accelerate intestinal absorption of glucose (18, 19), and all three agents can deplete the liver of glycogen (20–22) and uncouple oxidative phosphorylation (23, 24). However, the differences of these two phenols from thyroxine are well known, covering a range of effects from an inability to act as substitution therapy in myxedema (25, 26), to a failure to induce metamorphosis in the tadpole (27), to an inability to inhibit isolated dehydrogenases *in vitro* (28).

Nevertheless, the similarity in the alterations of the indices of thyroid function by these three agents (Table V) has led us to postulate that these changes are the consequence of some common action involving a reduction of the available TSH. What this action is remains to be discovered. The following possibilities have been considered.

It is tempting to correlate thyroid depression with the known ability of salicylate to uncouple oxidative phosphorylation, especially since dinitrophenol and thyroxine (23) are also uncoupling agents. On the basis of the dinitrophenol studies in the rat (4, 5), it had been postulated that the uncoupling action or the metabolic stimulation were possibly the factors common to agents that depress thyroid function via the pituitary gland. The present data make such a supposition less likely since a number of the dihydroxybenzoic acids that were found to depress I^{131} release from the rat thyroid (Table IV) neither raise the oxygen consumption in intact rats (32) nor uncouple

TABLE IV

The effect of salicylate and some of its congeners of the release of I^{131} from the rat thyroid

Compound	No. of rats	Dosage schedule	Biological half-life *before* drug	Biological half-life *on* drug	Ratio of half-life *on/off* drug	Comment
		mg./Kg. subcutaneously	*hours**	*hours*		
Salicylate	5	230 twice daily +0.9% in diet†	30	49	1.63	5% weight loss
(*o*-hydroxy-benzoate)	4	280 thrice daily	36	124	3.44	9% weight loss
	4	130 every 4 hrs.	24	68	2.83	6% weight loss
Salicylurate	4	490 twice daily†	50	46	0.92	9% weight loss
Salicylamide	6	280 thrice daily	21	31	1.48	Marked transient narcosis
m-hydroxy-benzoate	4	190 every 4 hrs.	36	36‡	1.00	Weight unchanged
p-hydroxy-benzoate	5	190 every 4 hrs.	25	25‡	1.00	Weight unchanged
Gentisate	5	150 every 4 hrs.	28	40	1.43	Weight unchanged
(2,5-dihy-droxy-ben-zoate)	5	320 every 4 hrs.	17	35	2.06	5% weight loss
γ-resorcyl-ate (2,6-di-hydroxyben-zoate)	5	160 every 4 hrs.	29	35	1.21	Weight unchanged
	5	320 every 4 hrs.	21	50	2.38	5% weight loss
α-resorcyl-ate (3,5-di-hydroxyben-zoate)	5	160 every 4 hrs.	23	31	1.35	Weight unchanged
	5	320 every 4 hrs.	23	36	1.56	Weight unchanged
	5	380 every 4 hrs.	27	46	1.70	Weight unchanged
2,4-dinit-rophenol	7	0.2% in diet	25	82	3.28	(4)
DL-thyroxine	5	0.075 daily	34	26 days	~18	(7)

* Variation of the release rate of control rats is in part caused by different feeding periods on the low iodine diets. Since each animal is its own control this error is eliminated.

† Low iodine diet supplemented with Purina Lab Chow. All other rats pretreated with low iodine diet alone.

‡ A change in half-life of under 10 per cent would not be detected by this technique.

oxidative phosphorylation at concentrations in excess of those found in the circulation (24).[2]

The observation that pyrogens or an elevated ambient temperature can depress thyroid function in rats as the body temperature is elevated (5) suggested that fever might be a factor in producing thyroid inhibition. However, the production of fever is not required since none of the patients described in the previous paper (1) had temperature elevation. Weight loss, though it can result from treatment with any of these agents and may be associated with a depression in the butanol-extractable iodine of serum (37), appears not to be essential since rats not exhibiting weight loss show similar thyroid depression [(5) and Table IV], and the salicylate-treated patients described in the previous paper (1) did not lose weight unless dietary intake was fixed. Moreover, patients on thyroid hormone therapy may

[2] Dr. Packer has kindly tested the 2,4-, 2,5-, 2,6-, dihydroxybenzoates and the meta- and parahydroxybenzoic acids in rat heart mitrochondria utilizing α-ketoglutarate, and has found that none of these phenols uncouple oxidative phosphorylation at concentrations as high as 2.5×10^{-3} M (33). It should also be pointed out that the uncoupling of thyroxine differs from the two other phenols. All three agents uncouple oxidative phosphorylation in intact mitochondria, but thyroxine fails to uncouple in mitochondria fragmented with digitonin, where dinitrophenol and salicylate are still active (34). Furthermore, uncoupling induced by thyroxine is sensitive to the concentration of Mg^{++}, whereas that of other phenols is not (35, 36).

TABLE V

Comparison of peripheral and thyroidal effects of thyroxine, salicylate and 2,4-dinitrophenol

	Thyroid hormone	Salicylate	2,4-dinitrophenol	References
Metabolic rate	↑*	↑	↑	
Urine creatine	↑	↑	↑	(1, 29, 30)
Serum cholesterol in hypothyroidism	↓	↓		(1, 26)
Glucose absorption	↑	↑		(18, 19)
Liver glycogen	↓	↓†	↓†	(20–22)
Oxidative phosphorylation	↓	↓	↓	(23, 24)
Thyroid I^{131} uptake	↓	↓	↓	(1, 4)
Thyroid I^{131} clearance	↓	↓		(1)
Release of thyroidal I^{131}	↓	↓	↓	(4, 7)
Serum protein-bound iodine	↓‡	↓	↓	(1–4)
Fractional rate of thyroxine disappearance from circulation	↑§	↑	↑	(1, 4, 31)
Goiter prevention	↑	↑‖	↑‖	(5)
Myxedema therapy	↑	0	0	(25, 26)
Tadpole metamorphosis	↑		↓	(27)
Dehydrogenase activity	↓	0	0	(28)

* ↑ refers to an increase or stimulation (or replacement in myxedema); ↓ refers to a decrease or inhibition; 0 means no effect.
† Initial depletion followed by an increase.
‡ After triiodothyronine treatment.
§ In thyrotoxicosis.
‖ Only partial.

exhibit thyroid inhibition in the absence of weight loss.

A theory of action that has been proposed for salicylates is that which emphasizes ortho-phenolic substitution and the chelation resulting from internal hydrogen bonding (17). Two possible effects might be involved: *a*) replacement of the hydrogen by some metal ion required for enzymatic reactions, and *b*) stabilization of the carboxylate anion leading to increased acidity, especially in the case of the di- ortho-substituted γ-resorcylate (38). The fact that gentisate and γ-resorcylate possess activity would be consistent with this formulation as would the inactivity of the *m*- and *p*-hydroxybenzoic acids (Table IV). However, 3,5-dihydroxybenzoic acid inhibits the release of I^{131} from the gland. Unless one would invoke conversion to an ortho-substituted product, it would seem unlikely that the chelation concept can be made to fit the data. The conversion to an active quinonoid intermediate (39) is also unlikely in this compound.

Since adrenal stimulation has been repeatedly suggested as a mechanism for salicylate action (40), it is important to consider this as a possible means of depressing thyroid function. Although the literature on the relation of the adrenals to the thyroid is extremely confusing (41), large doses of cortisone appear to be required in the rat to suppress iodine uptake, and only enormous doses (50 mg. per Kg. per day) depress release of I^{131} from the gland (42–45). Three mg. of cortisone per day did *not* prevent goiter formation (42). Furthermore, certain of the effects on the thyroid appear to be related to an augmented renal clearance of iodide (41) which did not occur in patients on salicylate therapy (1). That the adrenal is *not* required for dinitrophenol action was shown by the fact that goiter prevention by dinitrophenol still occurred in adrenalectomized rats maintained on constant parenteral hydrocortisone (5). In man treated with DNP, no change could be demonstrated in the eosinophile count or the urinary 17-ketosteroids (3). Finally, Peterson, Black, and Bunim (40) have shown that salicylate administration in man did not increase the plasma concentration or the rate of metabolism of hydrocortisone or corticosterone, or the urinary excre-

8894

SCHOOL OF DENTISTRY University of Detroit

tion of corticosteroids and 17-ketosteroids. An adrenal mechanism thus appears to be unlikely. Sensitization to the sympathomimetic amines is also not essential, since this is not a characteristic of salicylate-induced hypermetabolism (46).

Lastly, the evidence that both thyroxine and dinitrophenol (47) increase the disappearance rate of exogenous thyrotropin from the circulation does not afford a satisfactory explanation. If this were the reason for the inhibition of thyroid function, histologic evidence of pituitary activation might be expected. This, however, is not the case —both thyroxine and dinitrophenol treatment lead to normal or "inactive" pituitary cytology (5). Although a temporary rise in pituitary basophils had been reported following salicylate administration to rats (14), the cell type has not been identified as that characteristic of "thyrotrophs." Furthermore, the rise was not sustained, whereas the thyroid inhibition in man is demonstrable months after the onset of salicylate therapy.

It appears, therefore, that the TSH-inhibiting properties of the thyroid hormones can be mimicked by a number of agents that do not possess thyromimetic activity in many other respects, and that the mechanism of the inhibitory effect remains, at present, obscure.

SUMMARY

1. The effect of salicylate on some thyrotropin-dependent functions of the thyroid gland has been investigated in man and rats.

2. The thyroid of salicylate-treated patients responds to exogenous thyrotropin with an increase in uptake of I^{131}, an elevation of the serum protein bound iodine concentration, and an accelerated rate of release of I^{131} from the gland.

3. Salicylate treatment leads to partial inhibition of goitrogenesis in propylthiouracil-treated rats.

4. Salicylate-C^{14} does not appear to be significantly concentrated in the pituitary gland, hypothalamus, thyroid or adrenal.

5. Salicylate and a variety of its congeners retard the release of I^{131} from the rat thyroid gland.

6. It is concluded from these findings that the inhibition of thyroid gland function produced by salicylate therapy is mediated via an inhibition of thyrotropic stimulation at a pituitary or higher level. The mechanism of this effect has not been elucidated. It appears not to be related to the uncoupling of oxidative phosphorylation, pyrogenesis, chelating ability of ortho-substituted phenols, or adrenal stimulation.

7. A comparison of some actions of thyroxine, 2,4-dinitrophenol, and salicylate is presented.

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REFERENCES

1. Austen, F. K., Rubini, M. E., Meroney, W. H., and Wolff, J. Salicylates and thyroid function. I. Depression of thyroid function. J. clin. Invest. 1958, **37**, 1131.
2. Wolff, J., Rubin, L., and Chaikoff, I. L. The influence of 2,4-dinitrophenol on plasma protein-bound iodine. J. Pharmacol. exp. Ther. 1950, **98**, 45.
3. Castor, C. W., and Beierwaltes, W. The effect of 2,4-dinitrophenol on thyroid function in man. J. clin. Endocr. 1956, **16**, 1026.
4. Goldberg, R. C., Wolff, J., and Greep, R. O. The mechanism of depression of plasma protein bound iodine by 2,4-dinitrophenol. Endocrinology 1955, **56**, 560.
5. Goldberg, R. C., Wolff, J., and Greep, R. O. Studies on the nature of the thyroid-pituitary interrelationship. Endocrinology 1957, **60**, 38.
6. Goldsmith, R. E., Stanbury, J. B., and Brownell, G. L. The effect of thyrotropin on the release of hormone from the human thyroid. J. clin. Endocr. 1951, **11**, 1079.
7. Wolff, J. Some factors that influence the release of iodine from the thyroid gland. Endocrinology 1951, **48**, 284.
8. Bondi, S. Synthese der Salicylursäure. Z. physiol. Chem. 1907, **52**, 170.
9. Keller, W. J., Jr. A rapid method for the determination of salicylates in serum or plasma. Amer. J. clin. Path. 1947, **17**, 415.
10. Gerald, P. S., and Kagan, B. M. A method for the determination of gentisic acid in serum. J. biol. Chem. 1951, **189**, 467.
11. Roseman, S., and Dorfman, A. The determination and metabolism of gentisic acid. J. biol. Chem. 1951, **192**, 105.
12. Haberland, G. L., Medenwald, M., and Köster, L. Stoffwechselendprodukte der Salicyl-, Acetylsalicyl-, Gentisin- und Salicylursäure im Rattenurin. Hoppe-Seylers Z. physiol. Chem. 1957, **306**, 235.
13. Bogdanove, E. M., and Bogdanove, J. N. Thyroid weight, microscopic structure, and radioiodide concentration at various levels of thyrotropin secretion in the goitrogen-treated rat. Endocrinology 1957, **61**, 52.

14. Pallot, G., and Eberhardt, H. Distribution quantitative du salicylate marqué (C^{14}) dans l'organisme du rat. C. R. Soc. Biol. (Paris) 1956, **150**, 1220.
15. Meyer, K., and Ragan, C. The antirheumatic effect of sodium gentisate. Science 1948, **108**, 281.
16. Clarke, N. E., Mosher, R. E., and Clarke, C. N. Phenolic compounds in the treatment of rheumatic fever. Circulation 1953, **7**, 247.
17. Reid, J., Watson, R. D., Cochran, J. B., and Sproull, D. H. Sodium γ-resorcylate in rheumatic fever. Brit. med. J. 1951, **2**, 321.
18. Althausen, T. L., and Stockholm, M. Influence of the thyroid gland on absorption in the digestive tract. Amer. J. Physiol. 1938, **123**, 577.
19. Smith, M. J. H. The effect of salicylate on glucose tolerance in the rat. Biochim. biophys. Acta 1954, **14**, 241.
20. Barnes, J. M. The effect of dinitro-*o*-cresol on the deposition of liver glycogen in the rat. Biochem. J. 1953, **54**, 148.
21. Smith, M. J. H. The effect of salicylate on liver glycogen in the rat. Biochem. J. 1954, **57**, 349.
22. Fonnesu, A., and Severi, C. Glycogen accumulation in the liver due to 2:4-dinitrophenol. Brit. J. exp. Path. 1955, **36**, 35.
23. Lardy, H. A., and Maley, G. F. Metabolic effects of thyroid hormones *in vitro*. Recent Progr. Hormone Res. 1954, **10**, 129.
24. Brody, T. M. Action of sodium salicylate and related compounds on tissue metabolism *in vitro*. J. Pharmacol. exp. Ther. 1956, **117**, 39.
25. Dodds, E. C., and Robertson, J. D. The clinical applications of dinitro-o-cresol. Lancet 1933, **225**, 1137 and 1197.
26. Alexander, W., and Johnson, K. W. A comparison of the effects of acetylsalicylic acid and DL-triiodothyronine in patients with myxoedema. Clin. Sci. 1956, **15**, 593.
27. Cutting, C. C., and Tainter, M. L. Comparative effects of dinitrophenol and thyroxine on tadpole metamorphosis. Proc. Soc. exp. Biol. (N. Y.) 1933, **31**, 97.
28. Wolff, J., and Wolff, E. C. The effect of thyroxine on isolated dehydrogenases. Biochim. biophys. Acta 1957, **26**, 387.
29. Pugsley, L. I. The effect of 2:4-dinitrophenol upon calcium, creatine and creatinine excretion in the rat. Biochem. J. 1935, **29**, 2247.
30. Terroine, Th. Phénols nitrés et metabolisme azoté. Arch. int. Pharmacodyn. 1945, **71**, 377.
31. Sterling, K., and Chodos, R. B. Radiothyroxine turnover studies in myxedema, thyrotoxicosis, and hypermetabolism without endocrine disease. J. clin. Invest. 1956, **35**, 806.
32. Meade, B. W. Effect of certain hydroxybenzoic acids on the oxygen consumption of Wistar rats. Ann. rheum. Dis. 1954, **13**, 60.
33. Packer, L. Coupled phosphorylation in rat heart sarcosomes. Arch. Biochem. 1957, **70**, 290.
34. Lehninger, A. L. Personal communication.
35. Bain, J. A. The effect of magnesium upon thyroxine inhibition of phosphorylation. J. Pharmacol. exp. Ther. 1954, **110**, 2.
36. Mudd, S. H., Park, J. H., and Lipmann, F. Magnesium antagonism of the uncoupling of oxidative phosphorylation by iodo-thyronines. Proc. nat. Acad. Sci. (Wash.) 1955, **41**, 571.
37. Durham, J. R., Cooke, R. E., Lancaster, J. W., and Man, E. B. Serum butanol—extractable iodine values of children under ten years of age. Amer. J. Dis. Child. 1954, **87**, 468.
38. Baker, W. Abnormal strength of 2:6-dihydroxybenzoic acids. Nature (Lond.) 1936, **137**, 236.
39. Roof, B. S., and Turner, J. C. Protein interactions of gentisic acid and certain of its oxidation products. J. clin. Invest. 1955, **34**, 1647.
40. Peterson, R. E., Black, R. L., and Bunim, J. J. Salicylates and adrenocortical function in man. Arth. Rheum. 1958, **1**, 29.
41. Ingbar, S. H., and Freinkel, N. The influence of ACTH, cortisone, and hydrocortisone on the distribution and peripheral metabolism of thyroxine. J. clin. Invest. 1955, **34**, 1375.
42. O'Neal, L. W., and Heinbecker, P. Failure of cortisone to influence thyroid function. Endocrinology 1953, **53**, 239.
43. Perry, W. F. The action of cortisone and ACTH on thyroid function. Endocrinology 1951, **49**, 284.
44. Albert, A., Tenney, A., and Ford, E. The effect of cortisone and corticotropin on the biologic decay of thyroidal radioiodine. Endocrinology 1952, **50**, 324.
45. Brown-Grant, K. Inhibition of the release of thyroidal radioiodine in the rat by cortisone. Endocrinology 1955, **56**, 607.
46. Joy, R. J. T., and Austen, F. K. The response to catechol amine infusion during salicylate-induced hypermetabolism in man. Circulat. Res. In press.
47. D'Angelo, S. A. The metabolism of thyrotrophic hormone in the rat. Endocrinology 1955, **56**, 37.

THE FATTY ACIDS OF TOTAL LIPIDS AND CHOLESTEROL ESTERS FROM NORMAL PLASMA AND ATHEROMATOUS PLAQUES [1]

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In order to elucidate the mechanism of atheroma formation in arteriosclerosis, the lipid fractions of plasma and atheromatous plaques have been extensively studied in the past 45 years. It was first noted that there is a high content of cholesterol, phospholipid and total extractable lipids in atheromatous aortas (1–4). Then came a period when studies dealt primarily with the changing ratios between total, free and esterified cholesterol and phospholipids in normal plasma and atheromatous plaques (5–8). Considerable work has been done in the past decade to correlate the changes in blood cholesterol, phospholipids and lipoproteins with the incidence of arteriosclerosis (9–15). Comparatively little work has been done, however, on the isolation and identification of fatty acids associated with different lipid fractions of plasma and atheromatous plaques, the lack of adequate methods for fatty acid analysis being the most likely explanation. Schoenheimer (3) isolated the cholesterol esters of stearic, oleic and palmitic acids from the adventitia-free atheromatous aorta. In addition to these acids, McArthur (16) isolated linoleic and arachidonic acids from aortic plaques. As far as the fatty acids of the blood are concerned, the main sources of information are alkali isomerization studies (17–19) and studies done on the blood of large mammals (20). Recently, more information based on paper (21), column (22) and gas-liquid (23) chromatographic analysis of fatty acids of human blood has been reported. However, no comparative studies are available on material from human beings, using the same analytical methods to determine the fatty acids in blood and atheromatous plaques. There have been isolated reports suggesting that the blood of population groups having a low incidence of arteriosclerosis contains lipids of high iodine value (24). Another report has suggested that the feeding of fats with a high content of linoleic acid will raise the iodine value of the blood lipids, in addition to lowering the blood cholesterol (25). It has also been suggested that arteriosclerosis may develop as a result of essential fatty acid deficiency, or that the saturated acids, by virtue of their relative insolubility, may deposit preferentially in the arterial wall, thus playing an important role in the development of arteriosclerosis (26, 27). Since changes in blood cholesterol levels have been produced by feeding different fats, and speculations have been advanced as to the importance to be attributed to different types of fats in the production of arteriosclerosis (28–31), we have undertaken the present investigation in an effort to determine and compare the fatty acids of sterol esters and of total lipids (including triglycerides, cholesterol esters, phospholipids and free fatty acids) in normal plasma and atheromatous plaques, with the hope that such a study might shed light on the problem of atheroma formation.

MATERIALS AND METHODS

The lipids to be analyzed were obtained from normal blood and from atheromatous plaques. The fatty acids of total lipids and of cholesterol esters were first separated by counter-current distribution. The peaks were analyzed by means of alkali isomerization, determination of iodine value, gas-liquid chromatography, and paper chromatography before and after hydrogenation. The details are as follows:

Fasting venous blood from eight normal persons, who were considered to be in good health and good nutritional state and clinically free of arteriosclerosis and gross metabolic abnormalities, was collected in bottles containing sodium citrate as anticoagulant. Plasma was separated by centrifugation, pooled, and the total lipids extracted with 2:1 chloroform-methanol mixture (v/v) and purified according to the method of Folch and co-workers (32). The solvents were evaporated at 45° C. *in vacuo*

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in a flash evaporator (33). The total lipids were dissolved in a chloroform-methanol mixture. Aliquots were taken for the chromatographic separation of cholesterol esters, and for saponification with 15 per cent alcoholic KOH in order to obtain the fatty acids of total lipids. Saponification was carried out under an atmosphere of nitrogen, with refluxing, for five hours. Under these circumstances, no appreciable conjugation of poly-unsaturated fatty acids occurred.

Aortas were obtained from autopsy material. The intima was carefully separated from the media and outer layer, and the plaques were dissected. Most of the plaques were either calcified or ulcerated. They were cut into small pieces and then extracted with 2:1 chloroform-methanol mixture (v/v). For each gram of wet plaques approximately 20 ml. of solvent mixture was used. Extraction with the boiling solvent was continued for 30 minutes. The extraction was repeated four times with fresh solvent mixture. When aliquots of aortic plaques that had been extracted according to this method were hydrolyzed with ethanolic KOH solution, acidified and extracted with petroleum ether, no weighable amount of substance was obtained. The extracts were filtered, pooled and "purified" in a large water bath (32). The lipids were recovered from the extracts and treated further in the same way as described above for the lipids of plasma.

The chromatographic separation of sterol esters was done according to Borgström's method (34). The silicic acid [2] was used without prior activation. It was mixed with half its weight of Super-Cel,[3] and the slurry made in light petroleum ether (boiling range, 30 to 60° C.) was packed in columns of various sizes (2 to 8 cm. in diameter). The height of the columns was 9 to 10 cm. For a 2 cm. column, 300 mg. of total lipids was used, larger amounts being used with larger columns. The sterol esters were eluted with 8:2 light petroleum ether-benzene mixture (v/v).

The sterol esters were tested for contamination with other lipids as follows: Phosphorus was determined by the method of Lowry and associates (35). Free cholesterol was determined by the modified Schoenheimer-Sperry method (36). Free fatty acids were titrated with 0.01 N methanolic KOH. The upper limits of contamination, measured in these ways, were: free cholesterol, 0.3 per cent; phospholipid, 0.1 per cent; free fatty acids, 1 per cent. We had no satisfactory method for minute amounts of glycerol, and are unable to give meaningful figures for contamination with triglycerides. It was felt, however, that contamination with triglycerides was also quite small, since the total cholesterol content varied between 57.7 and 60.5 per cent, as determined by the method of Abell, Levy, Brodie, and Kendall (37) or by the modified Schoenheimer-Sperry (36) method. The theoretical cholesterol content, as calculated after the amounts of the various fatty acids present had been determined, was 59.9 per cent.

The cholesterol esters were saponified with alcoholic KOH in the same manner as the total lipids. The soaps from both of these sources were dissolved by heating in distilled water, and the unsaponifiable substances were removed by extraction five or six times with light petroleum ether. The soap solution was acidified with 50 per cent concentrated HCl in water and the fatty acids extracted three times with light petroleum ether. The extract was washed with distilled water until the water was neutral to litmus paper. It was dried with anhydrous sodium sulfate, and the solvents were evaporated *in vacuo*.

Separation and characterization of fatty acids: Countercurrent distribution. Distributions were carried out in a 200 cell, all glass, fully automatic machine described by Craig, Hausmann, Ahrens, and Harfenist (38). The solvent system used was a mixture of *n*-heptane, glacial acetic acid, formamide and methanol in the proportions 3:1:1:1 (39). About 1.5 to 2.5 Gm. of fatty acids was dissolved in lower phase previously equilibrated with upper phase, and distributed for about 420 transfers. The volumes of the upper and lower phases were each approximately 10 ml. After completion of the distributions, the concentrations of the fatty acids were determined by sampling the upper phase in every fourth tube. For complete recovery of the various acids, all of the solute in each tube was displaced into the upper phase by addition of water. The lower phase was then extracted three times with fresh *n*-heptane. The recoveries totalled 95 to 99 per cent of the weight of the acids distributed.

Fractions were recovered from the peaks and from different parts of the limbs of all curves for characterization and quantitation of the constituents present.

Iodine value. Iodine values were determined on 2 to 10 mg. samples, which were dissolved in 0.5 ml. of carbon tetrachloride and incubated for 45 minutes with 1 ml. of Wijs solution (40). After addition of 0.5 ml. of 15 per cent potassium iodide and 3 ml. of distilled water, the samples were titrated with 0.2 N thiosulfate solution, using an amperometric method suggested by Kolthoff and Bovey (41). All determinations were carried out in duplicate. The difference in the results of the duplicates was less than 1 per cent. The iodine values obtained on pure oleic and linoleic acids by this method agreed with values calculated from quantitative hydrogen uptake determinations done according to Pack, Planck, and Dollear (42).

Alkali isomerization. Alkali isomerization was done according to Herb and Riemenschneider (18). The recording of spectra between 230 and 380 mμ was done with a Model DK Beckman spectrophotometer.[4]

Paper chromatography. Paper chromatography of fatty acids was done on Whatman No. 3 paper impregnated with 10 per cent (v/v) liquid petrolatum, U.S.P.,

[2] Mallinckrodt Chemical Works, 100 mesh, analytical reagent, suitable for chromatographic analysis by the method of Ramsey and Patterson.

[3] Johns-Manville Corp.

[4] We are grateful to Mr. Page R. Edmondson for recording these spectra.

TABLE I

Retention volumes of methyl esters of various fatty acids

Compound	Retention vol. (V'R)*
	(*ml.*)
Methyl laurate	34
Methyl myristate	60
Methyl palmitate	94
Methyl stearate	167
Methyl arachidate	286
Methyl behenate	493

* Corrected for the pressure drop across the column. Conditions of chromatography as in Figure 1.

in ethyl ether. For short chain or highly unsaturated fatty acids, low concentrations (70 to 75 per cent) of acetic acid were used, and for longer chain or saturated fatty acids, higher concentrations (85 to 95 per cent). Staining of chromatograms was done according to Mangold, Lamp, and Schlenk (43). The unsaturated fatty acids were stained with iodine vapors. The chromatograms of saturated fatty acids were stained with iodine vapors after they had first been sprayed with 1 per cent α-cyclodextrin in 30 per cent ethanol.

Gas-liquid chromatography.[5] These analyses were carried out in a Podbielniak "Chromacon," Model 9475. The column was 4 mm. in diameter and 18 inches long, and consisted of 5 per cent silicone grease[6] on sodium chloride. The column temperature was kept constant at 234 to 235° C., and the sample heater temperature at 270° C. Helium gas, at a flow rate of 11.6 to 12.8 ml. per minute, was used for elution. The fatty acids were first esterified using diazomethane, prepared according to a standard method (44). The solution of diazomethane was added to an ethereal solution of fatty acids until a yellow color persisted. When the esterification was complete, the ether was evaporated and the methyl esters were dissolved in petroleum ether. Two and five-tenths to 4 μl. of solution containing 0.5 to 1 mg. of solute was placed on the column. The corrected retention volumes (V'R) were compared with those of known acids. Figure 1 shows the gas-liquid chromatographic analysis of a mixture of known saturated fatty acids from C_{12} to C_{22}. Table I shows the corrected retention volumes (V'R) of these acids. Under the conditions specified, the separatory power of this column, when calculated for stearic acid, was found to be 185 theoretical plates. With this column it was not possible to separate saturated and unsaturated fatty acids having the same chain length, although the retention volumes were not quite identical. In order to obtain analyses according to chain length

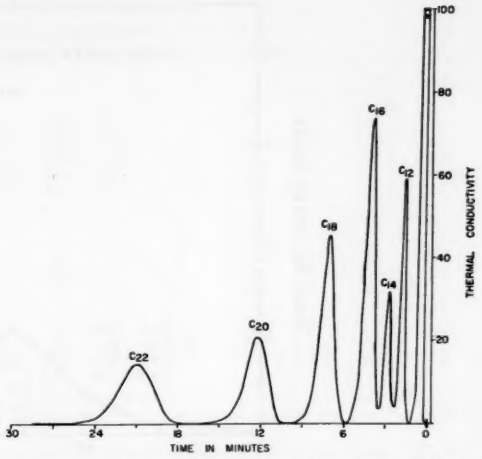

FIG. 1. GAS-LIQUID CHROMATOGRAM OF A KNOWN MIXTURE OF THE METHYL ESTERS OF LAURIC, MYRISTIC, PALMITIC, STEARIC, ARACHIDIC AND BEHENIC ACIDS

Sample size, 5 μl. (1 mg. fatty acids); column length, 18 inches, diameter (i.d.), 4 mm.; liquid phase, 5 per cent silicone grease on NaCl; column temp., 235° C.; flow rate of helium (at the inlet), 12.8 ml. per minute; inlet pressure, 110 mm. Hg; outlet pressure, atmospheric.

O, sample injection point; X, solvent peak.

The numbers on the ordinate are arbitrary units.

only, with complete elimination of the effect of unsaturation, some of the samples were hydrogenated and then analyzed by gas-liquid chromatography. The areas under the peaks were used to calculate the per cent composition of the samples examined, and the results were compared with those obtained from iodine value and alkali isomerization.

RESULTS

Fatty acids from total lipids of atheromatous plaques

One and seven-tenths Gm. of fatty acids obtained from the total lipids of pooled atheromatous plaques was subjected to counter-current distribution (Figure 2). The curve was divided into six fractions (Fractions A through F of Figure 2 and Table II). The contents of the tubes in each fraction were pooled, and the fatty acids were identified further by the methods discussed above.

Fraction D (tubes 100 through 143, less the peak tubes 123 through 127[7]*).* This fraction will

[5] We are indebted to Dr. D. H. Wheeler and Mr. J. Nelson of the General Mills Research Laboratories, Minneapolis, for the gas-liquid chromatographic analyses.

[6] Dow Corning high vacuum silicone grease was treated according to Cropper and Heywood (45).

[7] These numbers, as well as the tube numbers included in the figures and tables, represent the tube numbers in the apparatus. For the calculation of partition ratios, 200 must be added to all of them, since all of the solutes had made somewhat more than one full circuit.

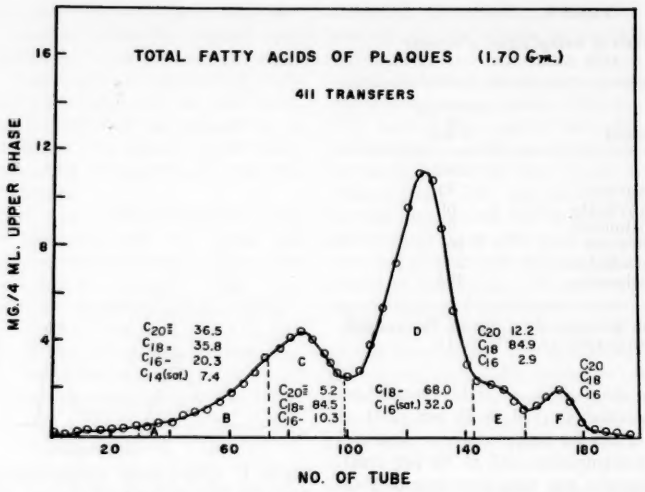

FIG. 2. COUNTER-CURRENT DISTRIBUTION CURVE OF THE FATTY ACIDS OF PLAQUES

The numbers in each fraction represent the per cent concentration of fatty acids as determined by gas-liquid chromatography.

be discussed first, because it is the largest fraction, and because the simplicity of its composition makes it particularly suitable for clarification of the manner of application of the various methods. The fraction consisted of a single peak by the counter-current method, incompletely separated from the adjacent peaks. Paper chromatography of this peak revealed a single spot with the Rf of palmitic and oleic acids. As pointed out by others (39, 43), however, these methods, both of which succeed principally on the basis of differences in polarity of the substances being separated, are subject to the following limitation: If the chain length of a fatty acid is increased by two carbon atoms, the polarity is decreased; if the number of double bonds is increased by one, the polarity is increased. These opposite effects are usually about equal, so that the mobility (Rf value) of a given fatty acid is about the same [8] as that of the acid with two more carbons and one more double bond. Gas-liquid chromatography, on the other hand, separates the fatty acids largely on the basis of molecular weight. In this respect it resembles distillation. The effect of double bonds is distinctly secondary. This method is capable of resolving the ambiguity remaining after application of the methods that achieve separation on the basis of polarity. The ambiguity may also be removed by hydrogenation of the fractions from the original counter-current distribution, followed by paper chromatography or a second run on the counter-current apparatus.

It is not surprising, in view of the above considerations, that Fraction D could be separated by gas-liquid chromatography into two peaks having the retention volumes of known fatty acids of chain lengths 16 and 18, respectively. Furthermore, after hydrogenation of the material, two spots appeared on paper chromatograms, and these had the Rf of saturated fatty acids with 16 and 18 carbons. The C_{18} acid must have had a single double bond in its native, unhydrogenated state, since it moved with the C_{16} saturated acid on paper and in the counter-current apparatus.

After alkali isomerization, the only absorption was a very small one at the wave length character-

[8] This generalization seems not to hold for arachidonic acid, which is poorly separated from linoleic acid. According to the generalization, arachidonic and linolenic acids should run together. In practice, when known mixtures of fatty acids are chromatographed, arachidonic acid appears between linoleic and linolenic acids, more nearly superimposed on linoleic acid.

TABLE II

*Fatty acids of atheromatous plaques**

Tube number	Fraction	Weight	Per cent of total	Iodine value, found	Iodine value, calculated†	Tetradecanoic ("Myristic")	Hexadecanoic ("Palmitic")	Hexadecenoic ("Palmitoleic")	Octadecanoic ("Stearic")	Octadecenoic ("Oleic")	Octadecadienoic ("Linoleic")	Octadecatrienoic ("Linolenic")	Eicosanoic‡ ("Arachidic") Eicosenoic Eicosadienoic	Eicosatetraenoic ("Arachidonic")
		mg.				*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
0– 30	A§	77.9	4.8	46.4										
31– 75	B	293.1	18.2	185.6	206.7	21.6	0	59.7	0	0	104.9	Trace	0	106.9
76– 99	C	228.5	14.2	161.0	182.9	0	0	23.1	0	0	189.9	0	0	15.3
100–143	D	614.5	38.1	63.7	65.6	0	179.4	0	0.	421.5	13.5	0	0	0
144–159	E	83.4	5.2	24.2		0	2.4	0	70.9	Trace	0	0	10.1	0
160–200	F‖	94.7	5.8	88.6										
82– 86	I	64.1	3.9			0	0	6.9	0	0	57.2	0	0	0
123–127	II	156.2	9.7			0	56.7	0	0	96.0	3.4	0	0.	0
Total mg.		1,612.4				21.6	238.5	89.7	70.9	517.5	369.0	Trace	10.1	122.2
Per cent of total		100				1.3	14.8	5.6	4.4	32.1	22.9	Trace	0.6	7.6

* The amount of fatty acids was calculated from data obtained by counter-current distribution and gas-liquid chromatography.

† The iodine values were calculated from the percentages of fatty acids determined by gas-liquid chromatography.

‡ The nature of these C_{20} acids is not yet definitely established. Preliminary investigations indicate that they are C_{20} saturated and C_{20} with probably one and two double bonds.

§ Unidentified; probably mostly oxidized fatty acids with trace amounts of C_{14}, C_{16}, and C_{18} acids, and minimal triene absorption.

‖ Unidentified; probably mostly polymerized fatty acids.

istic of a diene. It is reasonable to assume that this absorption was due to contamination from Fraction C.

Evidently Fraction D consisted mainly of a C_{16} saturated fatty acid (palmitic) and a C_{18} singly unsaturated fatty acid (presumably oleic), with a trace of diene (probably linoleic). Table II gives the composition of the fraction as determined by the combined techniques. The content of hexadecanoic (C_{16}, saturated) and octadecenoic (C_{18}, one double bond) acids was calculated from the gas-liquid chromatogram. The content of octadecadienoic acid (C_{18}, two double bonds, probably linoleic) was calculated from the alkali isomerization. The theoretical iodine value of the fraction, calculated from the above composition, was 65.6. The observed iodine value was 63.7. Thus, for this fraction, the methods employed were in satisfactory agreement.

Tubes 123 through 127, labeled Fraction II in Table II and comprising the peak tubes from Fraction D, were analyzed separately. The composition of the material in the peak did not differ greatly from that in the limbs of the curve. In addition to C_{16} saturated and C_{18} one double bond, alkali isomerization revealed 2.2 per cent dienoic acid within this fraction. Fatty acids other than C_{16} and C_{18} could not be demonstrated by paper chromatography after hydrogenation or by gas-liquid chromatography.

The fact that the percentage of dienoic acid was practically identical with that for Fraction D raises the possibility that a doubly unsaturated fatty acid of chain length greater than 18 carbons was present. One would not expect Fraction II to be so heavily contaminated with linoleic acid from Fraction C. If a longer chain dienoic acid was present, its concentration was too low to permit detection by paper chromatography after hydrogenation or by gas-liquid chromatography.

Fraction C (tubes 76 through 99 less tubes 82 through 86). According to the generalization mentioned above, this fraction, one step greater in polarity than Fraction D, might be expected to contain linoleic acid (C_{18}, two double bonds) and palmitoleic acid (C_{16}, one double bond). Paper chromatograms showed a spot corresponding in Rf to these, and also a spot with the Rf of arachidonic acid. Evidently the resolution attainable by paper chromatography in this region exceeds that of the counter-current technique as used in this study. Gas-liquid chromatograms revealed C_{16}, C_{18} and C_{20} acids, the main component being C_{18}. It is presumed that the C_{20} acid was arachidonic, poorly resolved in the counter-current apparatus from palmitoleic and linoleic acids.

Alkali isomerization confirmed these deductions. Eighty-one and one-tenth per cent of the fraction appeared to be a dienoic acid. By gas-liquid chromatography, 83.1 per cent was a C_{18} acid, presumably a diene in view of its mobility in the counter-current apparatus. Alkali isomerization indicated that 4.2 per cent of the fraction was a tetraenoic acid, and 0.95 per cent, pentaenoic. By gas-liquid chromatography, 6.7 per cent was C_{20} acids. Table V shows these comparisons for this fraction, as well as for all of the other fractions on which such comparative studies were carried out.

Similar measurements were done on tubes 82 through 86, taken from the peak of Fraction C and labeled Fraction I. Being further removed in the distribution from Fraction B, these peak tubes contained immeasurably small amounts of tetraenoic and pentaenoic acids (by alkali isomerization). Otherwise the composition was similar to that of Fraction C.

The quantitative data for Fractions C and I, taken from the gas-liquid chromatograms, are included in Table II.

Fraction B (tubes 31 through 75). According to the generalization about polarity, this fraction should contain arachidonic acid (C_{20}, four double bonds), as well as linolenic (C_{18}, three double bonds), if any is present, and myristic (C_{14}, saturated). In view of the lack of resolution from Fraction C, one would also expect to find the other components of that fraction. As set forth in Tables II and VI, the actual findings were in accord with these predictions. In addition, significant quantities of more highly unsaturated acids were present (by isomerization). Spectrograms obtained after alkali isomerization revealed absorption peaks at 232, 315, 345 and 374 mμ, indicating the presence of diene, tetraene, pentaene and hexaene acids. Table VII shows their quantities. There was no appreciable peak at 268 mμ (triene peak). After hydrogenation of the fraction, a small peak with the retention volume of a C_{22} acid appeared in the gas-liquid chromatogram. No such peak could be seen before hydrogenation, presumably because the C_{22} fraction consisted of several different highly unsaturated acids, each present in very small quantity and having slightly different retention volumes.

The presence of small amounts of C_{16} diene, in this fraction, cannot be excluded on the basis of our studies. Since the efficiency of our gas-liquid chromatography columns was rather low, and since counter-current distribution failed to separate the complex mixture of fatty acids present in this peak, the only method left was paper chromatography. Palmitolinoleic acid has an Rf value very close to that of linolenic acid. On paper chromatograms no iodine stainable spots could be detected that would suggest the presence of C_{16} diene. This would not, however, rule out the presence of such an acid, because paper chromatography, as used in this study, failed to detect fatty acids which constituted less than two per cent of a given mixture.

Fraction A (tubes 0 through 30). This portion of the counter-current distribution consisted mostly of an unidentified, amorphous material, which showed some degree of general absorption in the region of 270 to 275 mμ, before alkali isomerization, suggesting the presence of oxidized fatty acids (46). Because of lack of sufficient material, no other studies were carried out.

Fraction E (tubes 144 through 159). By gas-liquid chromatography, this fraction was largely C_{18} acids, with small amounts of C_{16} and C_{20}. In view of the low iodine value and the lack of an oleic acid spot on paper chromatograms, the principal component of this fraction was evidently stearic acid. Alkali isomerization showed the presence of 1.5 to 2 per cent of dienoic acid, probably due to a C_{20} acid with two double bonds.

Fraction F (tubes 160 through 200). This fraction was made up of an oily material with trace amounts of C_{16}, C_{18} and C_{20} acids. It showed several absorption bands in the ultraviolet after alkali isomerization. These bands may have been due to polymerized fatty acids. It has been demonstrated that in reversed phase paper chromatography the polymerized substances stay at the starting point of the chromatograms (47). In counter-current distribution, the polymerized substances would be expected to favor the less polar moving phase.

In the routine analyses no C_{12} acids were demonstrated. After preliminary vacuum distillation, however, trace amounts of C_{12} acids appeared in gas-liquid chromatograms from columns overloaded with the "C_{16}" fraction.

Infrared analyses for *trans* acids were carried out on various samples by Dr. J. R. Chipault, of

TABLE III

*Fatty acids of normal plasma**

Tube number	Fraction	Weight	Per cent of total	Iodine value, found	Iodine value, calculated†	Tetradecanoic ("Myristic")	Hexadecanoic ("Palmitic")	Hexadecenoic ("Palmitoleic")	Octadecanoic ("Stearic")	Octadecenoic ("Oleic")	Octadecadienoic ("Linoleic")	Octadecatrienoic ("Linolenic")	Eicosanoic‡ ("Arachidic") Eicosenoic Eicosadienoic	Eicosatetraenoic ("Arachidonic")
		mg.				*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
0–113	A	551.7	37.5	168.4	179.8	33.5	0	101.7	0	0	326.8	Trace	0	89.7
114–131	B	191.9	13.1	52.8	55.9	0	79.1	0	0	106.3	6.5	0	0	0
132–159	C	511.6	34.8	62.3	62.2	0	157.1	0	0	354.5	0	0	0	0
140–143	I	122.8	8.3			0	47.2	0	0	74.8	0	0	0	0
160–200	D§	91.0	6.2	74.9		0	3.4	0	83.2	0	0	0	4.4	0
Total mg.		1,469.0				33.5	286.8	101.7	83.2	535.6	333.3	Trace	4.4	89.7
Per cent of total		100				2.3	19.5	6.9	5.6	36.5	22.7	Trace	0.3	6.1

* The amount of fatty acids was calculated from data obtained by counter-current distribution and gas-liquid chromatography.

† See Table I.

‡ The nature of these C_{20} acids is not yet definitely established. Preliminary investigations indicate that they are C_{20} saturated and C_{20} with probably one and two double bonds.

§ The high iodine value in this fraction is most likely due to the oily, unidentified material (probably polymerized fatty acids). Since we do not know the per cent of this substance in the fraction, the actual amount of acids, calculated from gas-liquid chromatography data, may be somewhat lower than indicated in the table. However, this should not introduce a significant error in the final calculations, because Fraction D constitutes a small percentage of the total fatty acids. The oleic acid contamination should have been less than 3 per cent since on paper chromatography no oleic acid was detected. Alkali isomerization revealed 1.6 to 2 per cent diene absorption. C_{16}, C_{18} and C_{20} acids were found by gas-liquid chromatography.

the Hormel Institute. The *trans* content, expressed in terms of methyl elaidate, was 6.5 per cent for the fatty acids of plaques and 2.7 per cent for the fatty acids of normal plasma. The significance of these findings is not clear at this time.

Fatty acids from total lipids of normal plasma, from cholesterol esters of atheromatous plaques, and from cholesterol esters of normal plasma

Analyses on these three materials were carried out in a manner similar to that described above in detail for the fatty acids from total lipids of

TABLE IV

*Fatty acids of cholesterol esters of plaques**

Tube number	Fraction	Weight	Per cent of total	Iodine value, found	Iodine value, calculated†	Tetradecanoic ("Myristic")	Hexadecanoic ("Palmitic")	Hexadecenoic ("Palmitoleic")	Octadecanoic ("Stearic")	Octadecenoic ("Oleic")	Octadecadienoic ("Linoleic")	Octadecatrienoic ("Linolenic")	Eicosanoic‡ ("Arachidic") Eicosenoic Eicosadienoic	Eicosatetraenoic ("Arachidonic")
		mg.				*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
0– 70 181–200	A	533.1	18.3	202.6	226.0	17.6	0	86.9	0	0	204.7	Trace	?	224.5
71–105	B	950.2	32.7	154.0	180.0	0	0	103.9	0	0	791.5	0	0	54.7
106–160	C	1,186.4	40.8	72.0	73.5	0	237.3	0	0	925.2	23.6	0	0	0
161–180	D§	29.7	1.0	91.8					Present				Present	
85– 87	I	103.4	3.6			1.0	0	5.8	0	0	92.9	0	0	3.6
127–129	II	103.8	3.5			0	26.3	0	0	77.5	0	0	0	0
Total mg.		2,906.6				18.6	263.6	196.6	Present	1,002.7	1,112.7	Trace	Present	282.8
Per cent of total		100				0.6	9.1	6.8		34.5	38.3			9.7

* The amount of fatty acids was calculated from data obtained by counter-current distribution and gas-liquid chromatography.

† See Table I.

‡ The nature of these C_{20} acids is not yet definitely established. Preliminary investigations indicate that they are C_{20} saturated and C_{20} with probably one and two double bonds.

§ Mixture of octadecanoic, eicosanoic, eicosenoic and eicosadienoic. We have no quantitative data for oily material (probably polymerized fatty acids).

TABLE V

*Fatty acids of cholesterol esters of normal plasma**

Tube number	Fraction	Weight	Per cent of total	Iodine value, found	Iodine value, calculated†	Tetradecanoic ("Myristic")	Hexadecanoic ("Palmitic")	Hexadecenoic ("Palmitoleic")	Octadecanoic ("Stearic")	Octadecenoic ("Oleic")	Octadecadienoic ("Linoleic")	Octadecatrienoic ("Linolenic")	Eicosanoic‡ ("Arachidic") Eicosenoic Eicosadienoic	Eicosatetraenoic ("Arachidonic")
		mg.				*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
0– 80	A	377.2	25.4	197.4	202.0	15.1	0	44.6	0	0	223.6	Trace	0	93.9
81–108	B	275.4	25.3	160.7	175.0	0	0	23.9	0	0	351.5	0	0	0
109–148	C	421.1	28.4	57.8	57.0	0	150.4	0	0	281.4	0	0	0	0
149–176	D§	41.8	2.8	22.7		0	0	0	Present	Trace	0	0	Present	0
177–200	E‖	49.9	3.3											
86– 90	I	113.6	7.6	165.9		0	0	2.9	0	0	110.7	0	0	0
126–130	II	105.4	7.1			0	33.2	0	0	72.2	0	0	0	0
Total mg.		1,484.4				15.1	183.6	71.4	Present	353.6	685.8	Trace	Present	93.9
Per cent of total		100				1.0	12.4	4.8		23.8	46.2			6.3

* The amount of fatty acids was calculated from data obtained by counter-current distribution and gas-liquid chromatography.

† See Table I.

‡ The nature of these C_{20} acids is not yet definitely established. Preliminary investigations indicate that they probably are C_{20} saturated and C_{20} with one and two double bonds.

§ Forty-one and eight-tenths mg. equals 2.8 per cent mixture of octadecanoic, eicosanoic, eicosenoic and eicosadienoic. We have no quantitative data for these acids. Paper chromatography revealed trace amounts of oleic acid, in addition to stearic and C_{20} acids.

‖ Forty-nine and nine-tenths mg. equals 3.3 per cent unidentified, probably polymerized substance.

atheromatous plaques. The results are presented in Figures 3, 4 and 5 and Tables III through VII.

DISCUSSION

Methodology

At the present stage of their development, we feel that the various methods of lipid analysis should be used in combination, with cross checks at every possible opportunity. No single method currently available is capable of providing a complete analysis of the complex mixtures of fatty acids from biological sources. With the methods based on separation, the possibility that parts of the mixture remain unresolved is always present, unless the separated components are subjected to further identification. The isomerization method is not valid, even in theory, unless the identity and extinction coefficient of the various polyenes present are known, a criterion that is seldom fulfilled. In practice, the results by this method are even more dubious, because of the compounding of errors by the multiple subtractions of large quantities from each other. One's confidence is increased somewhat if one always runs complete

TABLE VI

Summary of fatty acid composition of the various lipids studied

Source of fatty acids	Tetradecanoic ("Myristic")	Hexadecanoic ("Palmitic")	Hexadecenoic ("Palmitoleic")	Octadecanoic ("Stearic")	Octadecenoic ("Oleic")	Octadecadienoic ("Linoleic")	Octadecatrienoic ("Linolenic")	Eicosatetraenoic ("Arachidonic")	Eicosanoic-Eicosenoic-Eicosadienoic ("Arachidic")
	%	%	%	%	%	%	%	%	%
Normal plasma	2.3	19.5	6.9	5.6	36.5	22.7	Trace	6.1	0.3
Plaques	1.3	14.8	5.6	4.4	32.1	22.9	Trace	7.6	0.6
Cholesterol esters (normal plasma)	1.0	12.4	4.8	X_1*	23.8	46.2	Trace	6.3	X_2*
Cholesterol esters (plaques)	0.6	9.1	6.8	X_3†	34.5	38.3	Trace	9.7	X_4†

* X_1 plus X_2 equals 2.8 per cent.

† X_3 plus X_4 equals 1.0 per cent.

TABLE VII

Comparison of the data obtained by gas-liquid chromatography (GLC) and alkali isomerization

Source	Fraction	Linoleic acid		Triene	Arachidonic acid		Pentaene	Hexaene
		G.L.C.	Isomerization "diene"*		G.L.C.	Isomerization "tetraene"†		
		%	%	%	%	%	%	%
Fatty acids of atheromatous plaques	A		2.3	1.8		1.1	1.5	1.9
	B	35.8	34.1	2.0	36.4	27.6	2.7	4.8
	C	83.1	81.1	0	6.7	4.2	0.9	0
	D		2.2	0	0	0	0	0
	I	89.1	84.9	0	0	0	0	0
	II		2.2	0	0	0	0	0
% of total		22.9	22.2	0.5	7.6	5.6	0.6	0.9
Fatty acids of normal plasma	A	59.2	53.6	3.3	16.3	16.3	2.3	3.8
	B		3.4	0	0	0	0	0
% of total		22.7	20.2	1.2	6.1	6.1	0.9	1.4
Fatty acids of cholesterol esters of plaques	A	38.4	36.4	2.8	42.1	34.7	3.4	3.7
	B	83.2	82.7	2.8	5.7	3.1	0.6	1.4
	C	0	1.9	0.5	0	0	0.2	0
	I	89.9	86.8	0	0	1.7	0	0
% of total		38.3	37.6	1.6	9.7	7.4	0.9	1.0
Fatty acids of cholesterol esters of normal plasma	A	59.3	52.8	1.9	24.9	24.5	2.8	4.6
	B	93.6	83.5	1.0	0	0	0	0
	I	97.4	86.3	0	0	0	0	0
% of total		46.2	41.1	0.7	6.3	6.2	0.7	1.2

* "Diene" calculated as linoleic acid.
† "Tetraene" calculated as arachidonic acid.

spectra, as was done in this work, rather than merely measuring the absorption at the specified wave lengths. In the present study, the results obtained by isomerization fitted quite well with those from the other methods. This agreement is probably due, at least in part, to the fact that the polyenes other than dienoic and tetraenoic acids were present in quite small concentration. Thus, the errors introduced into the estimation of the less highly unsaturated compounds due to errors in the measurement of the more unsaturated were minimal.

The rapid development of gas-liquid chromatography offers promise of eventually providing a single, rapid method for complete fatty acid analysis on small samples. As carried out in the cur-

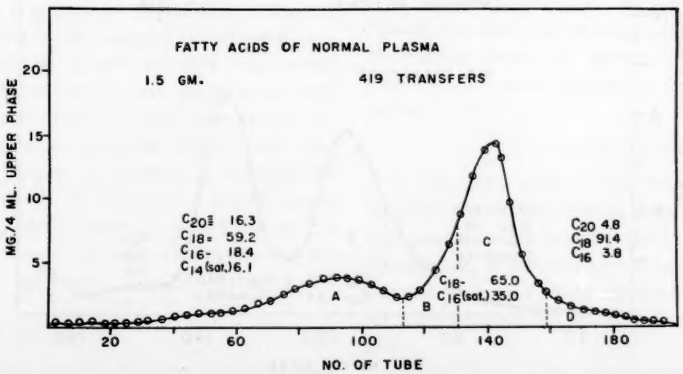

FIG. 3. COUNTER-CURRENT DISTRIBUTION CURVE OF THE FATTY ACIDS OF NORMAL PLASMA

See explanation under Figure 2.

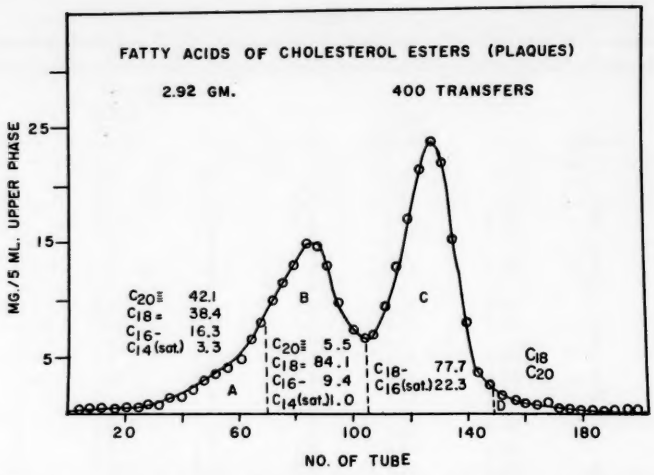

FIG. 4. COUNTER-CURRENT DISTRIBUTION CURVE OF THE FATTY ACIDS OF CHOLESTEROL ESTERS OF PLAQUES

See explanation under Figure 2.

rent study, the method suffered from the deficiency that accurate measurement of the amount of sample placed on the column was difficult, and the results had to be expressed as percentages of the total material recovered, rather than of that added. Furthermore, the resolving power of the column was insufficient to separate acids having the same chain length. It is our current practice to add an internal standard, to overcome the first of these deficiencies. Columns of much higher resolution have now been described, although preparation of such columns with regularity remains a difficult and highly empirical art.

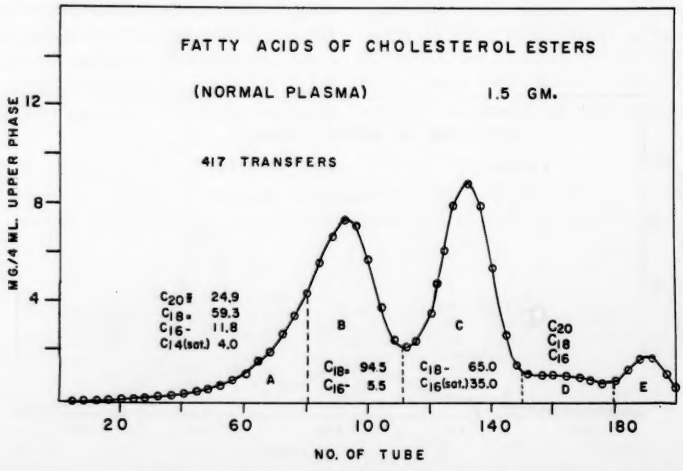

FIG. 5. COUNTER-CURRENT DISTRIBUTION CURVE OF THE FATTY ACIDS OF CHOLESTEROL ESTERS OF NORMAL PLASMA

See explanation under Figure 2.

Source of material

Some further comment is in order concerning the biological materials used. Because the methods required comparatively large quantities of fatty acids, the only practical approach was the pooling of samples from several individuals. The blood samples came from persons who were eating ordinary uncontrolled American diets. The effect of minor differences in these persons' dietary habits is unknown. The aortas were collected from persons of different ages, in whom the cause of death varied widely. We have no detailed information about their dietary habits, and we do not know the effect of such underlying diseases as diabetes mellitus, myocardial infarction, carcinoma, uremia, bacteremia and so forth. With improvement in the methodology, study of individual cases will be highly desirable.

Comparisons with other work

The finding of a high content of linoleic acid in cholesterol esters is in agreement with the work of Kelsey and Longenecker (20) for cow blood, and with that of Lipsky, Haavik, Hopper, and McDivitt (22) for human blood. James, Lovelock, Webb, and Trotter (23) were unable to demonstrate significant differences in the fatty acid composition of blood of persons with and without evidence of coronary artery disease. In view of our own findings, such a result would probably be anticipated.

The possibility that trace amounts of other fatty acids, as reported by James and associates (23), are present in the samples investigated cannot be excluded. It is quite possible that similar fatty acids are present in the plaques as well. Until the position of the double bonds in the unsaturated fatty acids isolated from blood and atheromatous plaques is known, a terminology which indicates only the number of double bonds and the carbon chain length is preferable to the more familiar names. In deference to custom, the familiar names were also included in this report.

Significance of this study

As noted in the introduction, we hoped to shed light on the mechanism of atheroma formation by this comparison of the fatty acid composition of plasma and of atheromatous plaques. Are the lipids deposited indiscriminately, regardless of their fatty acid composition, or are certain fatty acids particularly likely to be left behind as the lipoprotein molecules filter in and out of the arterial intima? Our analyses of the fatty acids of total lipids are compatible with the notion of indiscriminate deposition, but do not prove it because of the possibility that compensating differences exist in the various classes which make up the total lipids. In the case of the cholesterol esters, the ratio of linoleic acid to oleic acid was 1.9 in the plasma, but only 1.1 in the plaques. Because evaluation of the statistical significance of this difference is impossible, we prefer to defer further discussion of it, pending repetition of the analyses on individual patients. The following inference, however, does appear warranted: All of the various cholesterol esters of the plasma may be deposited in the arterial wall. None are specifically excluded. Although it is quite possible that population groups on different diets may have completely different patterns of fatty acids in the blood, it is difficult to conceive that those fatty acids would not be laid down if the proper conditions were present.

CONCLUSIONS

As summarized in Tables VI and VII, there appear to be neither qualitative nor gross quantitative differences between the total fatty acids of blood plasma and of atheromatous plaques. The linoleic acid of the cholesterol esters is relatively high in both blood and plaques. The ratio of linoleic to oleic acid is somewhat greater in the cholesterol esters of plasma than in those of plaques. No other significant differences are apparent.

SUMMARY

1. The fatty acids of the total lipids and the cholesterol esters of normal plasma and atheromatous plaques were investigated by counter-current distribution, determination of iodine value, alkali isomerization, gas-liquid chromatography, and paper chromatography before and after hydrogenation.

2. The fatty acids demonstrated were: saturated fatty acids C_{12}, C_{14}, C_{16}, C_{18} and C_{20}, and unsaturated fatty acids C_{16} with one and probably two double bonds, C_{18} with one, two and probably

three double bonds, C_{20} with two and probably more double bonds, and C_{22} with unknown numbers of double bonds. Fatty acids of unknown chain length with five and six double bonds were also present.

3. The octadecadienoic acid content of cholesterol esters was found to be higher than that of total lipids, both in blood and plaques.

4. No qualitative and gross quantitative differences were found between the total fatty acids of normal plasma and atheromatous plaques. The linoleic acid content of cholesterol esters of normal plasma was found to be higher than that of atheromatous plaques. The reverse was found for oleic acid. The significance of this finding is unknown.

5. The data were interpreted as support for the concept that plaques are formed by filtration and indiscriminate deposition of blood lipids in the arterial wall.

ACKNOWLEDGMENT

We wish to express our appreciation to Dr. Hermann Schlenk of the Hormel Institute, Austin, Minn., for advice on the techniques of paper chromatography of fatty acids, for a generous gift of *a*-cyclodextrin, and for carrying out the distillation of the methyl esters of the fatty acids of atheromatous plaques.

REFERENCES

1. Aschoff-Marburg, L. Ein Beitrag zur Myelinfrage. Verh. dtsch. path. Ges. 1906, **10**, 166.
2. Schoenheimer, R. Zur Chemie der gesunden und der atherosklerotischen Aorta. Hoppe-Seylers Z. physiol. Chem. 1926, **160**, 61.
3. Schoenheimer, R. Zur Chemie der gesunden und der atherosklerotischen Aorta. Hoppe-Seylers Z. physiol. Chem. 1928, **177**, 143.
4. Schoenheimer, R. 2. Mitteilung: Methodik zur quantitativen Trennung von ungesättigten und gesättigten Sterinen. Hoppe-Seylers Z. physiol. Chem. 1930, **192**, 77.
5. Meeker, D. R., and Jobling, J. W. A chemical study of arteriosclerotic lesions in the human aorta. Arch. Path. (Chicago) 1934, **18**, 252.
6. Zeek, P. M. A chemical analysis of atherosclerotic lesions in human aortas. Amer. J. Path. 1936, **12**, 115.
7. Weinhouse, S., and Hirsch, E. F. Chemistry of atherosclerosis. I. Lipid and calcium content of the intima and of the media of the aorta with and without atherosclerosis. Arch. Path. (Chicago) 1940, **29**, 31.
8. Page, I. H. Some aspects of the nature of the chemical changes occurring in atheromatosis. Ann. intern. Med. 1941, **14**, 1741.
9. Barr, D. P., Russ, E. M., and Eder, H. A. Protein-lipid relationships in human plasma. II. In atherosclerosis and related conditions. Amer. J. Med. 1951, **11**, 480.
10. Gertler, M. M., Garn, S. M., and Lerman, J. The interrelationships of serum cholesterol, cholesterol esters and phospholipids in health and in coronary artery disease. Circulation 1950, **2**, 205.
11. Gutman, A. B. Present status of atherosclerosis as a derangement of lipid metabolism. Amer. J. Med. 1953, **14**, 1.
12. Keys, A. Cholesterol, "giant molecules," and atherosclerosis. J. Amer. med. Ass. 1951, **147**, 1514.
13. Gofman, J. W., Jones, H. B., Lindgren, F. T., Lyon, T. P., Elliott, H. A., and Strisower, B. Blood lipids and human atherosclerosis. Circulation 1950, **2**, 161.
14. Gofman, J. W., Lindgren, F., Elliott, H., Mantz, W., Hewitt, J., Strisower, B., and Herring, V. The role of lipids and lipoproteins in atherosclerosis. Science 1950, **111**, 166.
15. Gofman, J. W., Glazier, F., Tamplin, A., Strisower, B., and De Lalla, O. Lipoproteins, coronary heart disease, and atherosclerosis. Physiol. Rev. 1954, **34**, 589.
16. McArthur, C. S. The acetone-soluble lipid of the atheromatous aorta. Biochem. J. 1942, **36**, 559.
17. Wiese, H. G., and Hansen, A. E. Semimicromethod for unsaturated fatty acids of blood serum. J. biol. Chem. 1953, **202**, 417.
18. Herb, S. F., and Riemenschneider, R. W. Spectrophotometric micromethod for determining polyunsaturated fatty acids. Analyt. Chem. 1953, **25**, 953.
19. Evans, J. D., Waldron, J. M., Oleksyshyn, N. L., and Riemenschneider, R. W. Polyunsaturated fatty acids in normal human blood. J. biol. Chem. 1956, **218**, 255.
20. Kelsey, F. E., and Longenecker, H. E. Distribution and characterization of beef plasma fatty acids. J. biol. Chem. 1941, **139**, 727.
21. Michalec, C. The nature of cholesterol esters in human blood serum. Biochim. biophys. Acta 1956, **19**, 187.
22. Lipsky, S. R., Haavik, A., Hopper, C. L., and McDivitt, R. W. The biosynthesis of the fatty acids of the plasma of man. I. The formation of certain chromatographically separated higher fatty acids of the major lipide complexes from acetate-1-C^{14}. J. clin. Invest. 1957, **36**, 233.
23. James, A. T., Lovelock, J. E., Webb, J., and Trotter, W. R. The fatty acids of the blood in coronary-artery disease. Lancet 1957, **272**, 705.
24. Snapper, I. Chinese Lessons to Western Medicine. New York, Interscience Publishers, Inc., 1941, pp. 30–31.
25. Shapiro, W. E., Estes, E. H., Jr., and Hilderman,

H. L. The effect of unsaturated fats on serum lipids in normal, active subjects. Clin. Res. Proc. 1957, **5**, 107.

26. Sinclair, H. M. Deficiency of essential fatty acids and atherosclerosis, etcetera. Lancet 1956, **270**, 381.
27. Gould, R. G. Lipid metabolism and atherosclerosis. Amer. J. Med. 1951, **11**, 209.
28. Ahrens, E. H., Jr., Tsaltas, T. T., Hirsch, J., and Insull, W., Jr. Effects of dietary fats on the serum lipides of human subjects (abstract). J. clin. Invest. 1955, **34**, 918.
29. Bronte-Stewart, B., Antonis, A., Eales, L., and Brock, J. F. Effects of feeding different fats on serum-cholesterol level. Lancet 1956, **270**, 521.
30. Beveridge, J. M. R., Connell, W. F., and Mayer, G. A. Dietary factors affecting the level of plasma cholesterol in humans: The role of fat. Canad. J. Biochem. 1956, **34**, 441.
31. Keys, A., Anderson, J. T., and Grande, F. "Essential" fatty acids, degree of unsaturation, and effect of corn (maize) oil on the serum-cholesterol level in man. Lancet 1957, **272**, 66.
32. Folch, J., Ascoli, I., Lees, M., Meath, J. A., and Le Baron, F. N. Preparation of lipide extracts from brain tissue. J. biol. Chem. 1951, **191**, 833.
33. Craig, L. C., Gregory, J. D., and Hausmann, W. Versatile laboratory concentration device. Analyt. Chem. 1950, **22**, 1462.
34. Borgström, B. Investigation on lipid separation methods. Separation of cholesterol esters, glycerides, and free fatty acids. Acta physiol. scand. 1952, **25**, 111.
35. Lowry, O. H., Roberts, N. R., Leiner, K. Y., Wu, M., and Farr, A. L. The quantitative histochemistry of brain. I. Chemical methods. J. biol. Chem. 1954, **207**, 1.
36. Sperry, W. M., and Webb, M. A revision of the Schoenheimer-Sperry method for cholesterol determination. J. biol. Chem. 1950, **187**, 97.
37. Abell, L. L., Levy, B. B., Brodie, B. B., and Kendall, F. E. A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. J. biol. Chem. 1952, **195**, 357.
38. Craig, L. C., Hausmann, W., Ahrens, E. H., Jr., and Harfenist, E. J. Automatic countercurrent distribution equipment. Analyt. Chem. 1951, **23**, 1236.
39. Ahrens, E. H., Jr., and Craig, L. C. Separation of the higher fatty acids. J. biol. Chem. 1952, **195**, 299.
40. Wijs Method, Official Methods of Analysis of the Association of Official Agricultural Chemists. Washington, D. C., Association of Official Agricultural Chemists, 1955, p. 465.
41. Kolthoff, I. M., and Bovey, F. A. Amperometric titration of Styrene with potassium bromate. Analyt. Chem. 1947, **19**, 498.
42. Pack, F. C., Planck, R. W., and Dollear, F. G. Determination of the total unsaturation of Tung oil by catalytic hydrogenation. J. Amer. Oil Chem. Soc. 1952, **29**, 227.
43. Mangold, H. K., Lamp, B. G., and Schlenk, H. Indicators for the paper chromatography of lipids. J. Amer. chem. Soc. 1955, **77**, 6070.
44. Diazomethane, Organic Syntheses, Collective Volume IIa, A. H. Blatt, Ed. New York, John Wiley & Sons, Inc., 1943, p. 165.
45. Cropper, F. R., and Heywood, A. Improvements in vapour-phase chromatography at relatively high temperatures. Nature (Lond.) 1954, **174**, 1063.
46. Holman, R. T., Lundberg, W. O., Lauer, W. M., and Burr, G. O. Spectrophotometric studies of the oxidation of fats. I. Oleic acid, ethyl oleate, and elaidic acid. J. Amer. chem. Soc. 1945, **67**, 1285.
47. Schlenk, H., Gellerman, J. L., Tillotson, J. A., and Mangold, H. K. Paper chromatography of lipides. J. Amer. Oil Chem. Soc. 1957, **54**, 377.

RED CELL CHANGES IN CHRONIC PULMONARY INSUFFICIENCY [1]

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It is well known that in chronic pulmonary disease there is often an increase in packed red blood cells. It is less widely recognized that while the hematocrit value rises, the hemoglobin concentration may remain normal. This is well shown in reports where both values are given (Table I). In 71 cases the mean hematocrit was 54.4 per cent, the mean hemoglobin was 15.8 Gm. per 100 ml., and the mean value for mean corpuscular hemoglobin concentration was 28.8 per cent (normal, 32 to 36 per cent). This is a study of the nature of the resulting hypochromia, which we have also consistently observed.

Methods for hemoglobin and hematocrit, when reported, varied in the papers cited. Figures for both venous and arterial blood are included. The inaccuracy of results of hemoglobinometry by acid or alkaline hematin conversion is well known, and has led to the recent widespread use of the cyanmethemoglobin method, while the venous hematocrit value may vary depending on the duration of tourniquet application as well as on the tube, centrifuge speed, and axis of rotation (1). It is hoped that in this study such inaccuracies have been avoided by the use of arterial blood oxygen capacity and uniform standard hematocrits. Blood oxygen capacity is a standard method for active hemoglobin; it does not include any inactive pigment which may be present. Comparison of the oxygen capacity (in ml. of oxygen per 100 ml. whole blood) to the hematocrit (in ml. of packed cells per 100 ml. whole blood) gives a ratio, the "$O_{2/Ve}$ ratio," of ml. of oxygen uptake per ml. of packed cells, which is the mean corpuscular *active* hemoglobin concentration. We use it in this study in place of the usual mean corpuscular hemoglobin concentration (MCHC) to emphasize that hemoglobin values are obtained gasometrically rather than colorimetrically, and because the question of inactive hemoglobin is part of the study. If inactive hemoglobin is neglected, MCHC can be derived from any $O_{2/Ve}$ ratio by dividing by 1.34 (ml. of O_2 taken up per gram of hemoglobin at standard temperature and pressure conditions). The mean normal value for oxygen capacity in our laboratory is 20.6 ± 0.95 volumes per 100 ml. and the mean normal hematocrit is 43.9 ± 1.87 per cent, giving an $O_{2/Ve}$ ratio of 0.469 ± 0.011. These values were found in eight healthy medical students.

The $O_{2/Ve}$ ratio in pulmonary disease has been found to be consistently reduced, and this appears to be due to an increase in red cell water.

TABLE I

Hematocrit and hemoglobin values from reported cases of chronic pulmonary disease

Source	No. of cases	Mean hematocrit	Mean hemoglobin	Mean corpuscular hemoglobin concentration
Normal values, Wintrobe (1)	28	47	16	34
Harvey (28)	28	51.1	14.1	27.6
Hammarsten (29)	16	54	16.8	32
Wilson (30)	14	52	15.2	29.6
Taquini (19)	6	69	20.1	29.3
Austen (31)	4	58	15.3	26.5
Carroll (32)	1	56	16	28.6
Loman and Dameshek (33)	1	80	22	27.5
Pare and Lowenstein (34)	1	68	19	27.9
Mean	71	54.4	15.8	28.8

METHODS

Arterial blood, collected anaerobically into heparinized syringes with mercury seals, was used for all measurements except for hematocrits. Oxygen and carbon dioxide content and oxygen capacity were determined by the method of Van Slyke and Neill. (2). The oxygen capacity is the volume of oxygen that can be liberated from blood after systematic equilibration with air for seven minutes in a tonometer, expressed in ml. per 100 ml. of whole blood. Arterial blood for hematocrits was ex-

[1] This work was supported in part by the Hitchcock Foundation.

pelled from dry syringes into mixed-oxalate tubes where it was gently and thoroughly agitated to prevent clotting. The hematocrit value was determined either by the standard method of Wintrobe (1) or by that of Strumia, Sample, and Hart (3); whole blood pH was determined anaerobically either by the glass electrode method of Behrmann and Fay (4) or by using a Cambridge research model pH meter with a jacketed electrode at 37° C. These methods have given interchangeable values in our hands. The partial pressure of carbon dioxide was determined from the carbon dioxide content, pH and hematocrit of the whole blood using the nomogram of Singer and Hastings (5). The partial pressure of oxygen was determined from the oxygen dissociation curves using the saturation and pH as given by Clark (6). Serum and whole blood water were determined according to Eisenman, Mackenzie, and Peters (7). Two ml. of whole blood or serum is weighed in a pyrex dish, then kept at 93 to 97° C. for 72 hours. Water content is taken as the original weight minus the final weight. Cyanmethemoglobin determinations were by the method of Drabkin (8) and those for carbon monoxide capacity were by that of Van Slyke, Hiller, Weisiger, and Cruz (9). Electrophoresis of hemoglobin solutions was performed by the method of Smith and Conley (10). Determinations of fetal hemoglobin were carried out on a Beckman D. U. apparatus by a modification of the method of White and Beaven (11). Solutions were denatured with 0.2 per cent NaOH, and the optical density at 575 millimicrons measured at 10 minute intervals. By this method mixtures of adult and umbilical cord blood give characteristic curves, depending on the proportion of cord blood.

Red cell diameter was measured on dried films photographed on Kodak metallographic plates projected to a magnification of 1,400; 200 cells were measured in two diameters at right angles to each other, each to the nearest 0.2 micron. The measuring error as determined by remeasurement of 100 cells was 0.0095 micron. Measurements in both patients and control subjects were by the same individual, who did not know the identity of the slides until after the results were reported. Statistical

TABLE II

Observed values for hematocrit and oxygen capacity in 31 males with chronic pulmonary disease

Patient	Hematocrit	O_2 Capacity	O_2/V_e	Age	Diagnosis
				yrs.	
Normal	43.9	20.6	0.469*	21	Eight healthy medical students
A. P.	58.0	20.8	0.359	47	†Bullous emphysema, fibrosis
H. M.	48.5	18.9	0.390	51	†Sarcoidosis, silicosis
J. G.	52.0	21.5	0.413	53	Emphysema, fibrosis
H. W.	43.4	17.7	0.408	62	†Emphysema, fibrosis
H. S.	51.7	23.0	0.445	51	†Emphysema, cor pulmonale
H. P.	60.5	23.6	0.390	54	†Bullous emphysema, fibrosis, cor pulmonale
G. LaR.	52.3	21.5	0.411	56	†Asthma, fibrosis, emphysema
B. S.	46.6	18.8	0.403	59	Emphysema, obesity
R. B.	53.9	23.2	0.430	59	Asthma, emphysema, fibrosis
F. S.	49.9	20.9	0.419	45	†Emphysema, fibrosis, cor pulmonale
C. McK.	53.1	21.3	0.401	33	Fibrosis, emphysema
L. G.	46.1	21.0	0.456	53	Emphysema, fibrosis
P. Y.	48.1	20.0	0.416	55	Asthma, emphysema
C. M.	49.1	21.2	0.432	48	Asthma, emphysema
C. L.	54.2	20.4	0.376	53	†Emphysema
E. W.	49.5	19.7	0.398	36	Fibrosis
R. R.	49.9	19.8	0.397	61	†Silicosis, emphysema, cor pulmonale
J. C.	45.2	19.0	0.420	37	Silicosis
E. S.	47.0	19.6	0.417	61	Asthma, emphysema, old thoracotomy with flail chest
R. W.	49.0	20.9	0.427	52	Bronchitis, emphysema, obesity
A. B.	56.8	23.1	0.407	41	Obesity, hypoventilation, cyanosis
R. H.	58.7	20.7	0.353	60	†Asthma, emphysema, cor pulmonale
F. T.	50.5	17.4	0.345	64	†Bronchitis, bronchiectasis, fibrosis
E. S.	54.1	16.9	0.312	55	Bullous emphysema, fibrosis, bronchitis
H. W.	60.0	22.7	0.378	63	†Emphysema, cor pulmonale
J. G.	54.6	21.4	0.392	55	Obesity, hypoventilation, cyanosis
J. C.	49.8	18.5	0.371	59	†Emphysema, cor pulmonale
A. S.	50.5	18.3	0.363	60	†Kyphoscoliosis, emphysema, cor pulmonale
J. C.	56.0	19.7	0.351	58	†Emphysema, fibrosis
L. J.	63.6	25.1	0.395	61	Bronchitis, fibrosis
B. W.	48.9	18.1	0.371	55	Emphysema, fibrosis
Mean	52.0	20.5	0.394‡	54	

* Range, 0.458 to 0.488.
† Confirmed by autopsy.
‡ Range, 0.312 to 0.456; S.D., 0.031; S.E., 0.006.

TABLE III

Hermatocrit and oxygen capacity in 22 patients without pulmonary disease

Name	Hematocrit	O_2 Capacity	O_{2/V_e}	Age	Diagnosis
				yrs.	
W. C.	40.8	18.1	0.444	36	Hypothyroidism
R. P.	43.0	19.5	0.452	23	Peptic ulcer
E. K.	46.8	20.9	0.447	29	Bullet wound in liver
W. S.	40.5	19.0	0.470	28	Infectious hepatitis
J. C.	44.6	19.8	0.444	40	Chronic lymphadenitis
J. M.	41.6	18.7	0.450	27	Infectious mononucleosis
R. B.	44.7	20.5	0.460	26	Conversion reaction
W. C.	44.6	19.2	0.430	39	Low back strain
L. N.	50.5	21.7	0.430	23	Flat feet
B. F.	46.6	20.4	0.438	40	Lipoma, right scapula
C. R.	41.6	18.9	0.453	26	Gastric neurosis
H. M.	46.9	21.9	0.466	29	Glomerulonephritis, latent
A. J. P.	28.0	12.8	0.457	59	Periarteritis nodosa
S. J. McC.	47.5	20.9	0.440	64	Nephrosis
G. T. S.	38.0	16.4	0.432	64	Carcinoma prostate
S. P.	42.0	16.8	0.424	66	Carcinoma colon
A. H.	36.0	15.9	0.442	70	Carcinoma prostate
F. S.	29.5	13.3	0.451	50	Glomerulonephritis
S. G.	45.5	21.1	0.469	45	Aortic stenosis
E. V.	40.5	17.6	0.435	70	Hiatus hernia
F. T.	40.0	17.8	0.445	63	Thoracic goiter
A. T.	50.5	22.5	0.446	46	ASHD; ? failure
Mean	42.3	18.8	0.447*	43	

* Range, 0.424 to 0.470; S.D., 0.013.

analyses were performed according to Bradford Hill (12) and Mainland (13).

RESULTS

The arterial hematocrit, oxygen capacity, and O_{2/V_e} ratio in 31 patients with chronic pulmonary disease are given in Table II, and contrasted to those obtained in eight healthy, 21 year old, male medical students. Usual clinical criteria for the diagnosis were supported by pulmonary function studies and in many instances by autopsy. The mean oxygen capacity was 20.5 ± 1.97 volume per cent in the patients and 20.6 ± 0.95 volume per cent in the controls; the mean hematocrit level was 52.0 ± 4.8 per cent in the patients and 43.9 ± 1.8 per cent in the controls. The mean O_{2/V_e} ratio in patients with pulmonary disease was 0.394 ± 0.031 and was 16 per cent below the normal value of 0.469. By the "t" test (13) p is less than 0.01, and the result is highly significant. Since these patients were on an average 33 years older than the normal controls, a group of 22 patients without pulmonary disorders (average age, 43) was contrasted to them (Table III). The ratio in patients with pulmonary disease is 12 per cent below the mean of 0.447 found in the control patients (p is again less than 0.01).

Attempts at correlation between measurements of O_{2/V_e} ratio and the arterial oxygen saturation, oxygen tension, pH, carbon dioxide content, and carbon dioxide tension (all of which varied from normal to grossly abnormal) were all unsuccessful. pH, carbon dioxide tension and oxygen tension are presented with the O_{2/V_e} ratios in Table IV. In general it was found that the ratio was consistently low when arterial oxygen unsaturation and respiratory acidosis (whether compensated or not) were of long standing.

Possible causes for the low O_{2/V_e} ratio that we investigated included the presence of inactive hemoglobin, abnormal hemoglobins, and rise in cell water concentration.

Inactive hemoglobin

In three cases we investigated inactive hemoglobin by the carbon monoxide capacity method of Van Slyke and co-workers (9) (Table V). Values found were less than 5 per cent and cannot account for the mean 12 per cent drop in O_{2/V_e} ratio. Comparison of the cyanmethemoglobin values with the hemoglobin values from oxygen capacity determinations also showed agreement within 5 per cent (Table VI). It is concluded that

TABLE IV

pH, carbon dioxide tension, oxygen tension, and O_{2}/V_{e} ratio in 30 males with chronic pulmonary disease

Name	pH	P_{CO_2}	P_{O_2}	O_{2}/V_{e}
		mm. Hg	*mm. Hg*	
"Normal"	7.43	41	100	0.469
A. P.	7.37	71	51	0.359
H. M.	7.35	42	40	0.390
J. G.	7.35	54	76	0.413
H. W.	7.41	58	39	0.408
H. S.	7.48	35	76	0.445
H. P.	7.47	49	47	0.390
G. LaR.	7.36	70	94	0.411
B. S.	7.36	51	42	0.403
R. B.	7.52	38	37	0.430
F. S.	7.43	49	54	0.419
C. McK.	7.48	45	37	0.401
L. G.	7.43	47	47	0.456
P. Y.	7.39	46	68	0.416
C. M.	7.42	48	51	0.432
E. W.	7.43	43	98	0.398
R. R.	7.50	55	34	0.397
J. C.	7.48	35	62	0.420
E. H. S.	7.44	44	59	0.417
R. W.	7.41	50	56	0.427
A. B.	7.42	42	56	0.407
R. H.	7.42	61	44	0.353
F. T.	7.42	58	35	0.345
E. S.	7.42	47	38	0.312
H. W.	7.32	69	22	0.378
J. G.	7.41	47	55	0.392
J. C.	7.41	64	23	0.371
A. S.	7.45	45	62	0.363
J. C.	7.33	61	49	0.351
L. J.	7.21	96	24	0.395
B. W.	7.31	69	22	0.371

the presence of inactive hemoglobin was not the explanation for the decreased oxygen capacity in the patients studied.

Abnormal hemoglobin

If fetal hemoglobin is present in a pigment solution, a characteristic notch at 289.8 millimicrons is produced in the curve of optical density. In hemoglobin solutions from eight of our patients with chronic pulmonary insufficiency, this notch was searched for but none was found. In the same eight cases denaturation of the pigment with 0.2 per cent NaOH gave curves which were uniformly typical of adult pigment (Figure 1). Abnormal pigments of types S, F, and C through I were searched for by filter paper electrophoresis

TABLE V

Inactive hemoglobin in chronic pulmonary disease, determined by carbon monoxide capacity

Subject	O_{2}/V_{e} ratio	Active hemoglobin	Total hemoglobin	Inactive hemoglobin
				%
F. T.	0.375	14.01	14.31	2.1
C. McK.	0.399	13.37	13.72	2.5
R. W.	0.429	13.88	14.51	4.3

TABLE VI

Inactive hemoglobin (fourth column) expressed as the difference between the values obtained by oxygen capacity and by cyanmethemoglobin conversion

Patient	Hemoglobin from cyanmet. Hgb.	Hemoglobin from O_2 cap.	Diff.
			%
R. H.	18.1	18.5	2
J. G.	12.5	12.6	1
G. A.	17.3	17.9	4
R. A.	14.7	15.5	5
H. C.	13.7	14.1	3
D. S.	15.5	16.0	3
R. G.	15.1	14.9	−1
E. G.	12.4	12.6	2

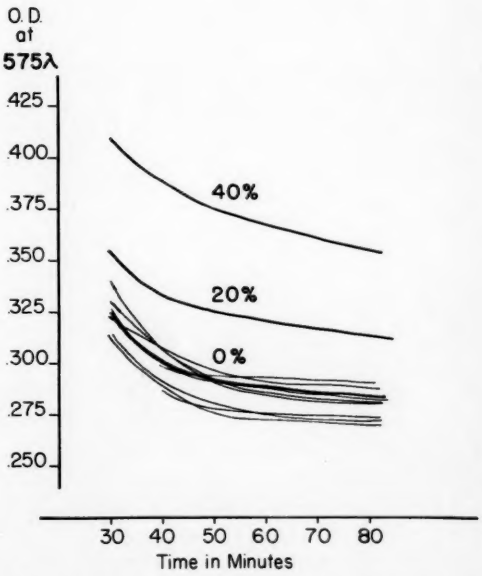

FIG. 1. ALKALI DENATURATION OF SOLUTIONS OF HEMOGLOBIN FROM EIGHT PATIENTS WITH CHRONIC PULMONARY INSUFFICIENCY (THIN LINES), CONTRASTED TO CURVES FROM CONCENTRATIONS OF UMBILICAL CORD BLOOD OF 40, 20 AND 0 PER CENT IN ADULT BLOOD

Negligible amounts of fetal hemoglobin are shown in patients by this method.

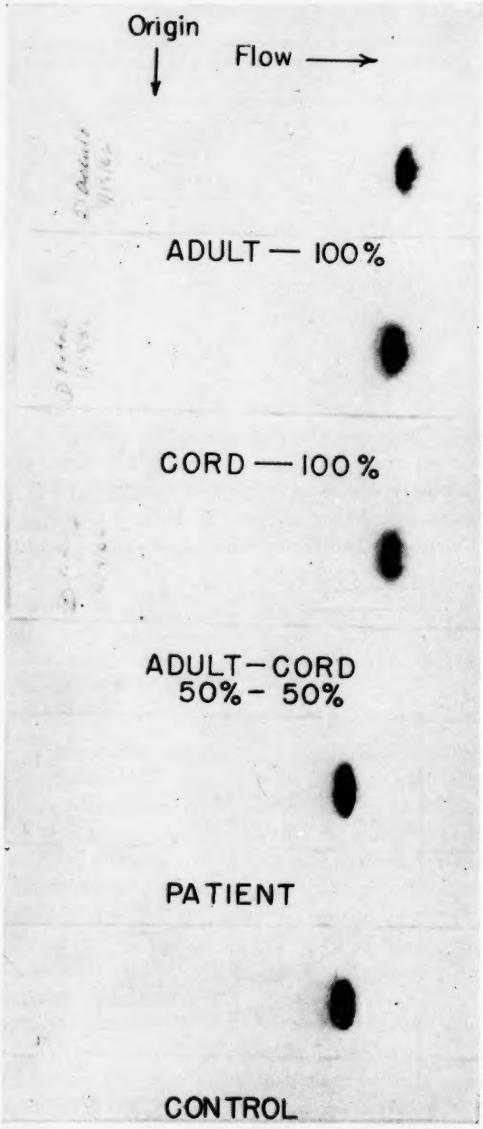

FIG. 2. ELECTROPHORETIC PATTERN OF PIGMENT MIGRATION IN PULMONARY DISEASE (FOURTH FROM TOP), IDENTICAL TO THE ADULT CONTROLS (BOTTOM)

Top patterns are those of umbilical cord and adult bloods as labeled.

after the method of Smith and Conley (10), and a typical pattern is shown in Figure 2. No abnormal pigment was found by the procedure.

Cell water

Decrease in hemoglobin concentration suggests a relative increase in other cell constituents, especially in water which constitutes about 72 per cent of the cell. Results of cell water determinations are shown in Figure 3, and it can be seen that in the 24 patients studied, cell water increase correlated well with the reduced O_{2/V_c} ratio. The coefficient of correlation was -0.95; standard error, 0.18. The extra water may be said to dilute rather than displace hemoglobin in the cell, since hemoglobin is not believed to be lost from intact human erythrocytes (14).

Increased packed cell water suggests but does not require an increase in cell size; the number of cells could vary independently. The mean red cell diameter for 9 healthy volunteers between ages 35 and 60 was 7.84 microns (S.D., 0.169) and for 10 patients with chronic pulmonary disease was 8.04 microns (S.D., 0.168; $p < 0.05$). The coefficients of variation are about 2 per cent. This shows a definite increase in red cell diameter in the patients with pulmonary disease. Mean frequency distribution curves of mean cell diameters are shown in Figure 4. The curve for the patients lies as a whole to the right, and shows a bulge or skew to the right in its shape. This was due to the presence of a double peak of frequency in six of the patients. The curves for all average diameter values to the right of the mean are shown in Figure 5. There are two peaks of frequency in the curves from patients with pulmonary insufficiency, one at 8.06 microns and the other at 8.16 microns, while the control curves show a single peak at 7.86 microns and a smooth descending limb.

DISCUSSION

In our patients the decrease in oxygen capacity compared to hematocrit was of the same order as that reported in the literature cited. The first explanation we considered was that plasma trapping, which has been reported to be relatively greater with high hematocrits (15), might be responsible for the increase in the hematocrit, with consequent reduction in the O_{2/V_c} ratio. The amount of trapped plasma in the red cell mass of the hematocrit tube has been carefully studied by different methods by Ebaugh, Levine, and Emerson (15) and by Chaplin and Mollison (16), and

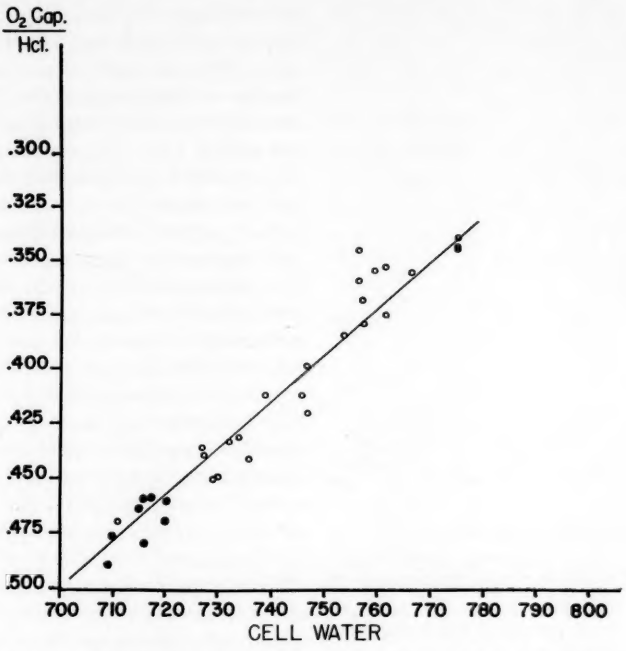

FIG. 3. CELL WATER (GRAM PER LITER OF PACKED CELLS) COMPARED TO $O_{2/Vc}$ RATIO

Black dots are normal males; open dots, patients with pulmonary disease. Coefficient of correlation, -0.95; S.E., 0.18. Regression equation: $(H_2O)\ c = 915 - 422\ O_{2/V}$.

they agree that at hematocrit levels of 50 per cent there is only about 2 to 2.5 per cent trapped plasma, not enough to explain the 16 per cent drop in $O_{2/Vc}$ ratio. Furthermore, if trapped plasma were the explanation the ratio should be decreased in direct and close proportion as the hematocrit rises, since plasma trapping varies directly with the hematocrit. This was not the case in our patients, in whom correlation between hematocrit and the $O_{2/Vc}$ ratio was only approximate, with a coefficient of correlation of -0.41 and a standard error of -0.18 (Figure 6). Finally, with the microhematocrit of Strumia, trapped plasma is negligible; yet the mean $O_{2/Vc}$ ratio in our cases studied with this apparatus was 0.381, compared to a mean of 0.403 in those whose hematocrit was determined by the method of Wintrobe. We conclude that plasma trapping is not the cause of the lowered ratio.

The next explanation to be considered was that the change in packed cell hemoglobin concentration might be a normal attribute of age, since the patients averaged over thirty years older than the medical students. Wintrobe (1), in his discussion of the erythrocyte, mentions no such phenomenon, stating only that with advanced age there may be a decrease in both hemoglobin and hematocrit. Robinson (17) found constant values for mean normal whole blood oxygen capacity from age 17 to 70 in males. Our own additional control data (Table III) are on 22 patients selected because of the absence of clinical pulmonary disease. There was a wide distribution of the $O_{2/Vc}$ ratio at all ages, ranging from age 23 to age 70. There was a poor negative correlation between age and the ratio, which decreased slightly as age increased (coefficient of correlation, -0.406; S.E., 0.22). The mean ratio in a group of 10 of the control patients of average age 60 was 0.444 ± 0.013, again highly significantly dif-

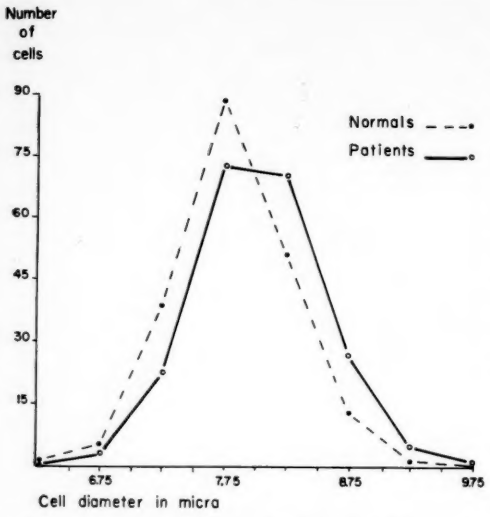

FIG. 4. MEAN FREQUENCY DISTRIBUTION CURVES OF CELL DIAMETERS, GROUPED AT 0.5 MICRON INTERVALS, FOR 10 PATIENTS WITH PULMONARY INSUFFICIENCY AND 9 CONTROLS OF COMPARABLE AGE

The curve for the patients lies as a whole to the right and shows a skew to the right.

ferent from that in the patients with pulmonary insufficiency ($p < 0.01$). Age alone, therefore, does not appear to explain the findings.

The possibility that a portion of the hemoglobin in these patients with chronic pulmonary disease was physiologically inactive appeared worth investigation. In a state characterized by chronic arterial oxygen unsaturation, hypercapnia and acidosis, the ability of red cell enzymes to reduce oxidized (ferric) hemoglobin to the ferrous functional form might be expected to be impaired, with consequent accumulation of methemoglobin and lowering of the numerator of the O_{2/V_c} ratio. Besides this possible alteration of the porphyrin complex of the hemoglobin molecule, the possibility that the globin portion might be abnormal was considered worth investigation. Several different types of pigment with abnormal globin moieties have now been reported (10) (S, F, and C through I), and denaturation of the globin may alter the reversible reaction between heme and oxygen. Though Meakins and Davies (18) and Taquini, Fasciolo, Suarez, and Chiodi (19) had found normal oxyhemoglobin dissociation curves in a few cases, the possible presence of abnormal pigment was considered worth further investigation. Fetal hemoglobin was of special interest since bone marrow in the fetus, as in patients with chronic pulmonary disease, functions at a low oxygen tension (20). Methods for fetal hemoglobin are of limited precision, but the failure of filter paper electrophoresis to yield any evidence of abnormal pigment is supported by the finding of no fetal pigment by spectrophotometry (Figure 1). The closeness of the results of hemoglobin determination by oxygen capacity to those of cyanmethemoglobin conversion and carbon monoxide capacity excludes the possibility of significant amounts of methemoglobin. (Table VI, incidentally, in emphasizing the close agreement between results of cyanmethemoglobin conversion and oxygen capacity, supports the value of the former for routine laboratory use.) An inactive pigment moiety, therefore, does not appear to explain the low O_{2/V_c} ratio.

Since water constitutes 72 per cent of red cells, and other constituents besides hemoglobin only 2 per cent, an increase in water would be the logical expectation if dilution of hemoglobin ex-

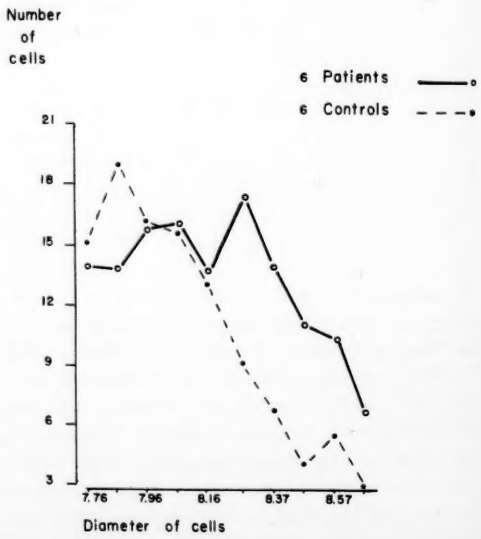

FIG. 5. FREQUENCY DISTRIBUTION CURVES FOR ALL CELL DIAMETERS 7.76 MICRONS AND ABOVE

The curve for the patients shows two peaks of frequency at 8.06 and 8.26 microns.

plains the $O_{2/Ve}$. Taquini and co-workers (19) found cell water per Gm. of packed cells to be elevated in three cases, with values of 0.740, 0.742, and 0.745 Gm. per ml. compared with a normal mean of 0.720, and related it to the decreased hemoglobin concentration. Our own studies on 32 individuals show good correlation between the increase in cell water above our normal value of 715 Gm. per L., and the decrease in $O_{2/Ve}$ ratio, both in healthy males and in patients (Figure 3). It is hard to escape the conclusion that the decrease in hemoglobin concentration results from dilution by the increase in cell water. The serum water, which Taquini found reduced, varied in our cases (range, 927 to 948 Gm. per L.).

Increased "cell water" values mean that the concentration of water in the packed cell column rises. If the number of cells remains constant, this would imply a rise in the individual cell water content, and consequently in cell volume. Berkson (21) has shown that hemocytometer cell counts, and hence values for mean cell volume that are derived from them, are determined significantly only to within plus or minus 12 per cent. One cannot therefore measure mean corpuscular volume within closer limits of accuracy. The most direct and accurate, although rather tedious, method of measuring cell size is that of Price-Jones (22). By photographing dried blood films, he found an increase in mean cell diameter from 7.20 in controls to 7.69 microns in 22 cases of emphysema. Frequency distribution curves were simply displaced to the right, retaining their shape, and he concluded that the enlarging effect was exerted on all circulating cells at once, and not on the hemopoietic organs.

Our own measurements confirm those of Price-Jones, in that the mean cell diameter was 0.2 micron greater in the 10 patients studied than in 9 controls. No correlation was found between individual mean cell diameter and the mean corpuscular hemoglobin concentration or the $O_{2/Ve}$ ratio, nor between cell diameter and the pO_2, oxygen saturation, oxygen capacity, pH, pCO_2, CO_2 content, or cyanmethemoglobin values. The overall mean increase appears nevertheless to be significant, both statistically and in the light of the small measuring error per 100 cells (0.0095 micron). The mean $O_{2/Ve}$ ratio in these 10 patients was 0.428, a 9 per cent decrease from normal.

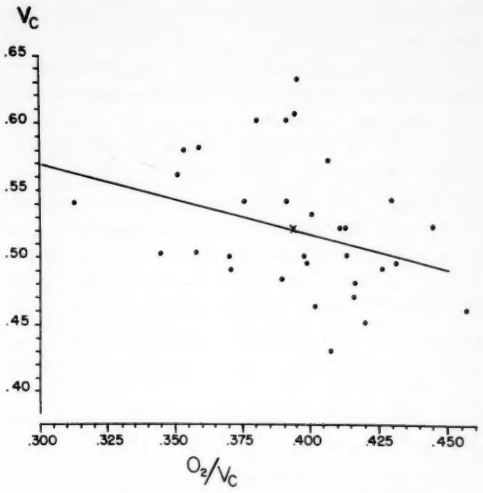

FIG. 6. CORRELATION BETWEEN $O_{2/VC}$ RATIO AND HEMATOCRIT

The relationship is only approximate; even though the correlation coefficient is more than twice its standard error, the data show a wide range of the ratio for any given hematocrit value.

According to Figure 3, this would correspond to a 3 per cent cell water rise. If the number of cells was constant, this would result in a mean 2 per cent volume increase, since the cell is 70 per cent water. While we have made no measurements of the thickness of the cells, it is usually taken as about 2 microns, and the increase that would result in cell diameter with such a volume change would be 0.08 micron, considering the cell as a short cylinder with a radius of 4 microns and a height of 2, and assuming no change in thickness. If such a cell with a diameter of 7.84 microns increases its volume 2 per cent by increasing its diameter alone, the new diameter can be calculated to be 7.92, while the diameter found was actually 8.04. It must be remembered that only rough agreement can be expected when one is comparing measurements from dried blood films with those from gravimetric and volumetric methods, and that no information is available as to changes in red cell thickness either in these patients, or in any disease states.

Our results differ from those of Price-Jones in that we recorded a double peak of cell diameter frequency in 6 of the 10 patients with emphysema.

No methodologic reason for this is apparent; the data for the controls were grouped by exactly the same process and no niches in the curves were found, and the choice of measurement intervals for cell grouping is shown not to be at fault since there is still a double peak when all the data are plotted (Figure 5). There appear to be two populations of cells in 6 of the 10 patients studied. Either they are produced by a change in only part of the normal adult population, or *de novo* by the marrow. We cannot tell from the curves which is correct. There was no relation between the presence of two frequency peaks and the $O_{2/Ve}$ ratio, or any of the other physiologic variables measured.

In vitro, it has been known since the early studies of Hamburger (23), von Limbeck (24), Henderson (25), and Van Slyke, Wu, and McLean (26) that increases in hematocrit, cell water and cell volume result when blood is exposed to increased carbon dioxide and low oxygen tensions. This is ascribed to the osmolar effects of increases in the concentration of cations and of bicarbonate and chloride in the cells when carbonic acid is buffered. The methods used in this study to determine oxygen capacity, hematocrit, cell water, and cell diameter all allow or encourage the free access of air to the blood under study, and allow roughly equal escape of carbon dioxide. (See Methods. We have measured the hematocrit and pH before and after air equilibration of seven blood specimens during the measurement of oxygen capacity; there was a mean drop of only 0.3 hematocrit unit with a rise in pH of 0.511 unit.) There does not therefore seem to be any methodologic cause for increased cell size. Also, since the bloods from the normal controls were handled in exactly the same manner, any such effect of carbon dioxide must have been applied equally to them.

We have failed to find correlation between the fall in $O_{2/Ve}$ ratio or the rise in cell water and the *in vivo* pO_2, pH or pCO_2. Fluctuations in the clinical state of the patients may perhaps explain this lack of correlation. Platts and Greaves (27), who have recently made careful measurements on the red cells in acute and chronic respiratory acidosis, have also found decreased concentrations of hemoglobin per volume of cell water. Their patients with chronic acidosis (pCO_2 over 52 mm. Hg) had somewhat increased red cell water concentration (741 Gm. per L. compared to their mean normal value of 722 Gm. per L.) and a mean cell pH of 7.21 compared to their normal 7.24. By contrast, normal subjects made acutely acidotic by breathing 7 per cent CO_2 showed no change in cell water despite a drop in cell pH from 7.24 to 7.12. *In vivo* findings in these studies, as in our own, failed to follow what would be predicted from the known *in vitro* behavior of blood, and suggest that the red cell response to acute CO_2 retention may differ from that in chronic acidosis. We have been unable to gauge accurately, in retrospect, the acuteness of the clinical state of pulmonary failure at the time of these measurements, but have the impression that the $O_{2/Ve}$ ratio was generally lowest in cases of longest standing.

SUMMARY

Patients with chronic pulmonary insufficiency show a rise in hematocrit but not in hemoglobin levels. This results in a lowered mean corpuscular hemoglobin concentration.

Studies of the arterial oxygen capacity and standard hematocrit values in 31 patients confirm this phenomenon in that the mean oxygen capacity: hematocrit ratio ("$O_{2/Ve}$ ratio") was 0.394, compared to the control value of 0.469.

The lowered ratio could not be correlated with pH, oxygen tension or saturation, or carbon dioxide tension or content.

Plasma trapping is not the explanation for the lowered ratio.

No inactive or abnormal hemoglobin was found in the blood to explain the decreased oxygen capacity.

A close inverse correlation was found between the ratio and the red cell water concentration, and it is concluded that entrance of water into the cells best explains the phenomenon.

The mean red cell diameter in 10 patients was found to be increased by 0.2 micron, which agrees roughly with the rise expected from the increased cell water.

REFERENCES

1. Wintrobe, M. M. Clinical Hematology, 4th ed. Philadelphia, Lea & Febiger, 1956.
2. Van Slyke, D. D., and Neill, J. M. Determination of gases in blood and other solutions by vacuum extraction and manometric measurement. J. biol. Chem. 1924, **61**, 523.

3. Strumia, M. M., Sample, A. B., and Hart, E. D. An improved microhematocrit method. Amer. J. clin. Path. 1954, **24**, 1016.
4. Behrmann, V. G., and Fay, M. A. A glass electrode vessel for the determination of blood pH. Science 1939, **90**, 187.
5. Singer, R. B., and Hastings, A. B. An improved clinical method for the estimation of disturbances of the acid-base balance of human blood. Medicine 1948, **27**, 223.
6. Clark, W. M. Topics in Physical Chemistry, 2nd ed. Baltimore, Williams and Wilkins, 1952, p. 204.
7. Eisenman, A. J., Mackenzie, L. B., and Peters, J. P. Protein and water of serum and cells of human blood, with a note on the measurement of red blood cell volume. J. biol. Chem. 1936, **116**, 33.
8. Crosby, W. H., Munn, J. I., and Furth, F. W. Standardizing a method for clinical hemoglobinometry. U. S. armed Forces med. J. 1954, **5**, 693.
9. Van Slyke, D. D., Hiller, A., Weisiger, J. R., and Cruz, W. O. Determination of carbon monoxide in blood and of total and active hemoglobin by carbon monoxide capacity. Inactive hemoglobin and methemoglobin contents of normal human blood. J. biol. Chem. 1946, **166**, 121.
10. Smith, E. W., and Conley, C. L. Filter paper electrophoresis of human hemoglobins with special reference to the incidence and clinical significance of hemoglobin C. Bull. Johns Hopk. Hosp. 1953, **93**, 94.
11. White, J. C., and Beaven, G. H. A review of the varieties of human haemoglobin in health and disease. J. clin. Path. 1954, **7**, 175.
12. Hill, A. B. Principles of Medical Statistics. London, The Lancet, 1948.
13. Mainland, D. Elementary Medical Statistics. Philadelphia, Saunders, 1952.
14. Shemin, D., and Rittenberg, D. The life span of the human red blood cell. J. biol. Chem. 1946, **166**, 627.
15. Ebaugh, F. G., Jr., Levine, P., and Emerson, C. P. The amount of trapped plasma in the red cell mass of the hematocrit tube. J. Lab. clin. Med. 1955, **46**, 409.
16. Chaplin, H., Jr., and Mollison, P. L. Correction for plasma trapped in the red cell column of the hematocrit. Blood 1952, **7**, 1227.
17. Robinson, S. Experimental studies of physical fitness in relation to age. Arbeitsphysiologie 1938, **10**, 251.
18. Meakins, J. C., and Davies, H. W. Respiratory Function in Disease. Edinburgh, Oliver and Boyd, 1925.
19. Taquini, A. C., Fasciolo, J. C., Suarez, J. R. E., and Chiodi, H. Respiration and circulation in pulmonary anoxemia. Arch. intern. Med. 1948, **82**, 534.
20. Barcroft, J. Fetal circulation and respiration. Physiol. Rev. 1936, **16**, 103.
21. Berkson, J. Blood Cell Count: Error *in* Medical Physics, O. Glasser, Ed. Chicago, Year Book Publishers, 1944, vol. I, pp. 110–114.
22. Price-Jones, C. Red Blood Cell Diameters. London, Oxford Medical Publications, 1933.
23. Hamburger, H. J. Über den Einfluss der Athmung auf die Permeabilität der Blutkörperchen. Z. Biol. 1891, **28**, 405.
24. von Limbeck, R. Ueber den Einfluss des Respiratorischen Gaswechels auf die rothen Blutkörperchen. Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak. 1895, **35**, 309.
25. Henderson, L. J. Blood: A Study in General Physiology. New Haven, Yale University Press, 1928.
26. Van Slyke, D. D., Wu, H., and McLean, F. C. Studies of gas and electrolyte equilibria in the blood. J. biol. Chem. 1923, **56**, 765.
27. Platts, M. M., and Greaves, M. S. The composition of the blood in respiratory acidosis. Clin. Sci. 1957, **16**, 695.
28. Harvey, R. M., Ferrer, M. I., Richards, D. W., Jr., and Cournand, A. Influence of chronic pulmonary disease on the heart and circulation. Amer. J. Med. 1951, **10**, 719.
29. Hammarsten, J. F., Whitcomb, W., Lowell, J., and Johnson, P. C. Red cell life span and iron turnover in patients with hypoxia due to pulmonary emphysema. Amer. Rev. Tuberc. In press.
30. Wilson, R. H., Borden, C. W., and Ebert, R. V. Adaptation to anoxia in chronic pulmonary emphysema. Arch. intern. Med. 1951, **88**, 581.
31. Austen, F. K., Carmichael, M. W., and Adams, R. D. Neurologic manifestations of chronic pulmonary insufficiency. New Engl. J. Med. 1957, **257**, 579.
32. Carroll, D. Peculiar type of cardiopulmonary failure associated with obesity. Amer. J. Med. 1956, **21**, 819.
33. Loman, J., and Dameshek, W. Plethora of the intracranial venous circulation in a case of polycythemia. New Engl. J. Med. 1945, **232**, 394.
34. Pare, P., and Lowenstein, L. Polycythemia associated with disturbed function of the respiratory center. Blood 1956, **11**, 1077.

AN HEREDITARY ENZYMATIC DEFECT IN ERYTHROCYTE METABOLISM: GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY [1]

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Alterations in glutathione metabolism have been detected in the erythrocytes of patients with hemolytic anemia induced by ingestion of a variety of chemical agents including primaquine (1), acetanilid, sulfanilamide (2), naphthalene (3), fava beans (4) and nitrofurantoin (5). Erythrocytes sensitive to these agents may be identified by an *in vitro* test in which incubation with acetylphenylhydrazine leads to loss of reduced glutathione. Employing this test, it was found that among individuals without a previous history of hemolytic anemia, the incidence of subjects with drug sensitive erythrocytes was approximately 4.8 per cent in Negroes and apparently rare in Caucasians (6). The incidence and racial distribution of this defect in glutathione metabolism was similar to the previously reported distribution of primaquine sensitivity (7).

The reactions involved in the maintenance of glutathione in the reduced state may be illustrated by the following scheme [2] (8–10):

$$\text{1. G-6-P} + \text{TPN}^+ \xrightleftharpoons{\text{G-6-P.D.}} \text{6-phosphogluconolactone} + \text{TPNH} + \text{H}^+$$

$$\text{2. 6-phosphogluconolactone} \xrightarrow{\text{Lactonase}} \text{6-PG.}$$

$$\text{3. 6-PG.} + \text{TPN}^+ \xrightleftharpoons{\text{6-PG.D.}} \text{R-5-P} + \text{CO}_2 + \text{TPNH} + \text{H}^+$$

$$\text{4. GSSG} + \text{TPNH} + \text{H}^+ \xrightarrow{\text{GSSG red.}} 2\text{GSH} + \text{TPN}^+$$

Carson, Flanagan, Ickes, and Alving (10) have demonstrated that the addition of 6-PG., but not G-6-P, to hemolysates of primaquine sensitive erythrocytes could initiate the above reactions leading to GSH formation. These authors interpreted their findings as indicating that the basic abnormality in red blood cells of primaquine sensitive subjects was a deficiency in G-6-P.D. Recently, Waller, Löhr, and Tabatabai (11) reported an absence of G-6-P.D. and an accumulation of TPN in the erythrocytes of one patient with chronic hemolytic anemia of undetermined etiology.

In the present study a reduction in erythrocyte G-6-P.D. has been demonstrated in healthy subjects without a history of hemolytic disease as well as in patients who have had a drug induced hemolytic anemia. This investigation establishes that this enzymatic defect in erythrocyte metabolism is on an hereditary basis.

METHODS

Subjects studied. 1. Control subjects (Table I). The control subjects were selected from a group of individuals in good health and without a history of known hematological disease. Among 227 subjects tested, 13 were found to have a deficiency of G-6-P.D. and were excluded. The controls, those without deficiency of G-6-P.D., then comprised a group of 214 subjects, 98 Negroes and 116 Caucasians. Fifty-six of this group were newborn infants (20 Caucasians and 36 Negroes).

2. Subjects without known hematological disorder but with a deficiency of erythrocyte G-6-P.D. This group

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[2] The following abbreviations are used: G-6-P., glucose-6-phosphate; G-6-P.D., glucose-6-phosphate dehydrogenase; TPN, triphosphopyridine nucleotide; 6-PG., 6-phosphogluconate; 6-PG.D.,-6-phosphogluconic dehydrogenase; TPNH, reduced triphosphopyridine nucleotide; R-5-P, ribulose-5-phosphate; GSSG, oxidized glutathione; GSH, reduced glutathione; GSSG red., glutathione reductase; PNP, purine nucleoside phosphorylase.

included the 13 subjects discovered among the 227 healthy individuals tested (Table II). Four were Negro infants, 7 Negro adults, and 2 Caucasian adults. Family studies were obtained in 5 of these subjects: K. R.—father, mother and one sibling; W. C.—father, mother and five siblings; Baby F.—mother and father; M. H.—husband and four children; M. A.—one child. All the members of the families were in good health and none gave a history of hemolytic anemia.

3. Family studies of subjects with a history of naphthalene induced hemolytic anemia (Table III). Four weeks prior to study, Subject R. A. had hemolytic anemia with a history of moth ball ingestion. Family studies of this individual included the mother and father, one male and two female siblings. All members of this family were well at the time they were studied, except the mother who was hospitalized because of an incomplete abortion associated with an anemia secondary to blood loss. Subject Mi. P. was studied one year following an acute episode of naphthalene induced hemolytic anemia. Family studies included the mother, father, one female and two male siblings, all of whom were well. R. B. was a subject who, two years prior to study, had an acute episode of naphthalene induced hemolytic anemia. Family studies included the mother, one female sibling and one male sibling, all of whom were well. Subject Do. C. had a history of naphthalene induced hemolytic anemia two years prior to study. Family studies included the maternal grandmother, paternal aunt, one female sibling, one male nonidentical twin, two male maternal half-siblings, and one female maternal half-sibling, all of whom were well. I. O. T. experienced naphthalene induced hemolytic anemia six years prior to study. Family studies included the mother, one female sibling and one male maternal half-sibling, none of whom had a history of hemolytic anemia.

4. Family studies of subjects with fava bean induced hemolytic anemia (Table IV). J. P. had a history of favism eight years prior to study. Family studies included the mother and one female maternal half-sibling, both of whom were well. Subject L. C. gave a history of favism eight years prior to this study. Family observations included the father (German) and mother (mixed European extraction) who were both well. D. D., an Italian female, had a history of favism five years ago. Family studies included her husband, two sons and one married daughter. The daughter's husband and two children, one male and one female, were also included. None of the family members gave a history of hemolytic anemia. K. K., a 12 year old female of Greek extraction, experienced favism seven years prior to the present work-up. In her family, in which three generations were available for study, it was possible to test 37 blood relatives and 8 spouses of these relatives. Her father was dead as a result of a coronary occlusion and was not known to have had hemolytic anemia. In her immediate family, her mother, one male and three female siblings, one brother-in-law, one niece and one nephew were all well and were studied. Her mother had two male and two female siblings, all of whom denied having had hemolytic anemia. One uncle, unmarried, was studied. The other uncle refused study but his wife and three male and two female children were tested. All were well. One aunt and her male child were studied, the latter had a history of hemolytic anemia induced by sulfanilamide administration 20 years previously. The other aunt, her husband, and five male and two female children were studied. Four of the children were married. Their spouses and their 12 children were studied. All were well.

TABLE I

Glucose-6-phosphate dehydrogenase (G-6-P.D.), 6-phosphogluconic dehydrogenase (6-PG.D.), purine nucleoside phosphorylase (PNP) and reduced glutathione (GSH) in erythrocytes of control subjects

Assay	Number of subjects	Adults: Caucasians	Adults: Negro	Mean ± S.D.
G-6-P.D.*	158	96	62	16.2 ± 2.2
6-PG.D.*	78	44	34	17.0 ± 3.8
PNP*	49	26	23	145.0 ± 20.8
% GSH protected	16	13	3	89.0 ± 10.0
Neonatal period†				
G-6-P.D.*	56	20	36	20.4 ± 3.3
6-PG.D.*	22	7	15	21.8 ± 3.7
% GSH protected 0–55 hrs. old	20	7	13	32.0 ± 10.0
% GSH protected 55–92 hrs. old	8	3	5	73.0 ± 7.1

* Enzyme units are expressed as ΔO.D. per minute per Gm. hemoglobin.

† Neonatal period includes full term infants of one week of age and younger.

5. Family studies of subjects with hemolytic anemia of unknown etiology (Table V). Baby T. had a mild hemolytic anemia with no evidence of blood group or Rh incompatability. Family studies included mother, father and two female siblings who were all well. Subject H. W. had an acute episode of hemolytic anemia of undetermined etiology two and one-half months previously. Family studies included the father, mother and one male sibling who were well.

Procedures. The techniques employed for the assay of erythrocyte enzyme activity have been previously described (12). Five ml. of heparinized venous blood was centrifuged for 10 minutes at 1,500 × G. The plasma and buffy coat were removed and the erythrocytes washed twice in an isotonic potassium chloride solution buffered at pH 7.4. The red blood cells were resuspended in approximately two volumes of this potassium chloride solution. The preparation of erythrocytes was performed at 0° C. Aliquots of the resuspended cells were lysed by freezing and thawing twice. Assays for G-6-P.D., 6-PG.D. and P.N.P. were performed on these hemolysates employing methods (13–15) with modifications that have been described elsewhere (12). All enzyme activities

TABLE II

Studies of individuals with deficient erythrocyte glucose-6-phosphate dehydrogenase, without known hematological disorders, and their families

Subject	Age*	Sex	Race	G-6-P.D.	6-PG.D.	PNP	Initial GSH	GSH protected	Hgb.
				ΔO.D./min./Gm. Hgb.			*mg./Gm. Hgb.*	%	*Gm./100 ml.*
Baby C. L.	30 days	M	N	2.5					12.5
K. R.	8 mos.	M	N	2.3	10.8				
	9 mos.			2.3	12.3		0.6	28	11.5
Mother R.	31 yrs.	F	N	7.8	13.7		1.2	47	12.6
Father R.	35 yrs.	M	N	1.7	12.8		1.0	15	15.5
Brother R.	10 yrs.	M	N	20.7	20.0		1.6		10.8
W. C.	14 mos.	M	N	5.7	24.2		1.6	22	9.3
Mother C.	29 yrs.	F	N	9.5	15.2		1.1	75	12.9
Father C.	31 yrs.	M	N	11.3	14.1		1.6	93	14.6
Sister C.	11 yrs.	F	N	11.8	13.2		1.4	80	13.8
Sister C.	10 yrs.	F	N	7.0	10.7		1.2	66	13.8
Sister C.	9 yrs.	F	N	12.0	14.0		1.6	95	14.0
Brother C.	6 yrs.	M	N	2.7	9.6		0.8	13	12.1
Brother C.	4½ yrs.	M	N	13.8	15.8		1.6	84	11.1
Baby F.	2 days	F	N	8.3	12.2		2.1	25	16.7
	4 days			8.9	13.9		1.7	57	16.4
Mother F.	22 yrs.	F	N	15.0	18.2		1.3	100	12.0
Father F.	21 yrs.	M	N	0.7	9.3		0.6	41	14.6
Mother H.	36 yrs.	F	N	7.4			1.2	72	13.8
Father H.	35 yrs.	M	N	12.3	10.0		1.2	81	17.4
Son H.	11 yrs.	M	N	12.3			1.4	67	13.4
Daughter H.	10 yrs.	F	N	12.8			1.0	94	13.0
Son H.	13 yrs.	M	N	2.0	9.5		0.6	12	12.7
Son H.	14 yrs.	M	N	13.2			1.0	89	11.9
Mother A.	33 yrs.	F	W	7.2			1.8		15.5
Baby A.	2 days	M	W	18.1	18.1		1.9	72	18.2
G. W.†	26 yrs.	F	N	7.4	10.2		0.8		10.8
P. P.†	33 yrs.	M	N	6.2	12.3	140			16.7
C. McM.†	30 yrs.	M	N	4.4	11.1	125			15.9
M. W.†	63 yrs.	M	W	6.6	16.0	131			13.5
B. D.†	70 yrs.	M	N	3.4	13.5	174			14.6
A. T.†	33 yrs.	M	N	6.1	12.4	150			12.3
C. M.†	70 yrs.	F	N	6.6	14.2	160			11.4

* Age at time of study.
† Family studies not obtained.

were expressed as the change in optical density units per minute per gram of hemoglobin (Δ O.D. per minute per Gm. Hgb.).

Reduced glutathione was measured by the method of Grunert and Phillips (16). The change in erythrocyte concentrations of GSH following a two hour incubation with acetylphenylhydrazine was employed as the basis for determining erythrocyte GSH stability according to the procedure of Beutler (6). Hemoglobin concentrations were measured by pipetting a suitable quantity of hemolysate into 10 ml. of 0.4 per cent NH_4OH and reading the optical density of the resulting solution in a spectrophotometer at 540 mμ.

RESULTS

Table I summarizes the control data for erythrocyte concentrations of the enzymes, G-6-P.D., 6-PG.D. and PNP and values of the GSH stability test. Tables II through V present the details of the studies performed on subjects with a deficiency in erythrocyte G-6-P.D. and their families.

Glucose-6-phosphate dehydrogenase

Decreased levels of erythrocyte G-6-P.D. were found in three categories of individuals: *1*) 4 Negro infants discovered in the course of testing 135 infants (31 Negro and 54 Caucasian),[3] and

[3] Erythrocytes from a total of 135 infants were assayed for G-6-P.D. activity. Four of these had significantly low levels of enzyme, leaving a total control group of 131. In only 56 of these infants were hemoglobin assays performed on the hemolysates used for enzyme analysis

TABLE III

Family studies of individuals with naphthalene hemolytic anemia

Subject	Age*	Sex	Race	G-6-P.D.	6-PG.D.	PNP	Initial GSH	GSH protected	Hgb.
				ΔO.D./min./Gm. Hgb.			*mg./Gm. Hgb.*	*%*	*Gm./100 ml.*
R. A.†	14 mos.	F	N	4.1	20.7		1.9	25	11.3
	15 mos.			1.5	12.7		1.7	7	11.8
Mother A.	24 yrs.	F	N	9.1	15.6		1.8	54	8.2
				7.0	16.3		1.5	56	11.8
Father A.	25 yrs.	M	N	13.7	14.7		1.6	100	16.5
Brother A.	4 yrs.	M	N	2.4	12.3		1.3	17	10.6
Sister A.	6 yrs.	F	N	8.0	13.2		1.3	62	12.4
Sister A.	8 yrs.	F	N	16.3	17.4		0.8	92	19.8
Mi. P.†	3 yrs.	M	N	4.5	13.3	152			13.3
Mother P.	35 yrs.	F	N	3.6	12.6	141			12.2
Father P.	37 yrs.	M	N	17.4	12.6	126			13.9
Brother P.	7 yrs.	M	N	2.4	13.4	147			12.0
Brother P.	5 yrs.	M	N	2.5	13.6	131			12.5
Sister P.	10 yrs.	F	N	10.9	10.2	143			13.8
R. B.†	3 yrs.	M	N	3.4	13.0	159			13.4
Mother B.	29 yrs.	F	N	13.3	13.3	134			13.5
Sister B.	4 yrs.	F	N	21.0	22.6	154			
Brother B.	1 yr.	M	N	5.0		196			13.0
Do. C.†	5 yrs.	M	N	2.7	12.9	157			13.0
Grandmother	63 yrs.	F	N	16.0	18.9	105			13.6
Paternal aunt	49 yrs.	F	N	19.1	16.1	186			12.0
Sister C.	8 yrs.	F	N	6.6	18.1	147			13.9
Twin C.	5 yrs.	M	N	19.7	17.6	149			13.2
Half-brother	4 yrs.	M	N	17.8	16.7	131			13.6
Half-brother	2 yrs.	M	N	20.1	18.1	121			13.8
Half-sister	3 yrs.	M	N	17.3	17.2	131			13.4
I. O. T.†	8 yrs.	M	N	1.5			0.8	20	12.2
Mother T.	35 yrs.	F	N	2.0			0.7	40	13.1
Half-brother T.	16 yrs.	M	N	1.5	9.7		0.7	28	16.1
Sister T.	2 yrs.	F	N	6.6	11.7		0.8	61	13.8

* Age at time of study.
† Propositus who had hemolytic anemia.

9 adults (7 Negro and 2 Caucasian) discovered in the course of testing 170 adults (71 Negro and 99 Caucasian), none of whom had known hematological disorders; *2*) 8 Negro and 3 Caucasian individuals known to have had hemolytic anemia —5 following naphthalene ingestion, 4 after fava bean consumption, and 2 of undetermined etiology; *3*) 39 (26 Negro and 13 Caucasian) relatives of the above individuals. One Caucasian male in this group of relatives gave a history of sulfonamide induced hemolytic anemia. A total of 103 (49 Negro and 54 Caucasian) relatives were studied.

Among the 52 subjects with a decreased erythrocyte G-6-P.D. content but no history of hematological disease, 24 had moderate reductions in G-6-P.D. All values in this group were between 2.5 and 4.0 standard deviations from the control mean. The mean value for the intermediate group was 8.9 Δ O. D. per minute per Gm. Hgb. with a standard deviation of ± 1.3. Of these 24 individuals, 23 were females. The remaining 28 subjects, and 12 of the 13 with a history of hemolytic anemia, had levels of erythrocyte G-6-P.D. which fell below 4 standard deviations of the mean for the control subjects. The mean value for the group with low enzymes was 2.75 Δ O. D. per minute per Gm. Hgb., with a standard deviation of ± 2.0.

so as to provide the necessary data to express the units of enzyme activity per gram of hemoglobin. In the other 75 infants, the enzyme concentrations were referred to a volume of packed erythrocytes and, therefore, could not be used to compare with the control adult data or the data on the subjects with G-6-P.D. deficiency. Accordingly, the control data as presented in Table I include only 56 infants. However, for a correct impression as to the incidence of the G-6-P.D. deficiency, the four infants with a deficiency in erythrocyte G-6-P.D. without known hematological disorder must be considered in relation to a total group of 135.

TABLE IV

Family studies of individuals with favism

Subject	Age*	Sex	Race	G-6-P.D.	6-PG.D.	Initial GSH	GSH protected	Hgb.
	yrs.			*ΔO.D./min./Gm. Hgb.*		*mg./Gm. Hgb.*	*%*	*Gm./100 ml.*
J. P.†	12	M	N	1.9	11.3	1.3	14	12.9
Mother P.	21	F	N	9.1	14.9			
Sister P.	6	F	N	2.4	9.8			
D. D.†	50	F	W	0.4	12.6	0.8	6	15.8
Husband B. D.	64	M	W	12.6	15.2	0.9	98	17.0
Son Do. D.	32	M	W	0.1	10.5	0.4	22	19.5
Son A. D.	22	M	W	0.1	12.0	0.4	30	15.8
Daughter A. L.	28	F	W	9.0	14.0	1.0	93	14.1
Husband L.	30	M	W	14.9	10.9			15.0
Son J. L.	6	M	W	0.1	12.0	0.6	15	13.4
Daughter E. L.	4	F	W	9.0	16.0	1.1	74	13.2
L. C.†	12	M	W	0.4	10.5	0.6	19	15.2
Mother C.	21+	F	W	8.2	14.0	1.6	75	13.9
Father C.	21+	M	W	16.5	17.0	1.3	96	15.9
K. K.†	12	F	W	7.8	7.6	1.0		14.8
Mother K.	50	F	W	7.6	10.0	1.3	64	13.6
Brother T. K.	14	M	W	15.0	10.0	1.1		16.7
Sister N. K.	21	F	W	11.0	11.0	0.7		14.0
Sister A. K.	19	F	W	14.1	14.7	1.1		13.8
Sister A. H.	23	F	W	8.6	16.3	0.8		14.1
Husband R. H.	25	M	W	12.6	10.0	1.4		15.0
Daughter D. H.	2	F	W	14.3	15.6	0.9	100	12.2
Son B. H.	4	M	W	11.7	12.9	0.9	100	13.9
Maternal uncle W. S.	40	M	W	12.2	9.8	0.9	100	16.0
Maternal aunt M. D.	52	F	W	8.7	9.9	1.3	91	12.5
Son A. D.‡	25	M	W	0.0	11.7	0.6	34	12.5
Maternal aunt A. S.§	60	F	W	10.9		1.3	86	14.4
Daughter A. S.	20	F	W	11.4		1.4	95	13.9
Daughter K. S.	28	F	W	11.8		1.5	95	13.7
Son T. S.	25	M	W	10.9		1.2	98	16.6
Son B. S.	24	M	W	10.7		0.9	93	16.7
Son E. S.	27	M	W	12.1		0.6	47	16.5
Maternal aunt E. K.	59	F	W	0.0	11.1	0.8	23	13.8
Husband A. K.	60	M	W	13.9		1.7	100	12.6
Son A. K.	30	M	W	0.0		0.9	13	15.3
Wife S. K.	22	F	W	14.5		1.5	83	13.2
Daughter L. K.	1	F	W	11.5		1.6	67	12.8
Son P. K.	21	M	W	12.3		1.6	88	16.8
Son J. K.	19	M	W	11.3		1.1		15.7
Son G. K.	26	M	W	12.5		1.6	81	16.8
Wife E. K.	22	F	W	16.0		1.6	89	14.0
Daughter V. K.	6	F	W	13.8		1.3	89	14.3
Daughter L. K.	5	F	W	13.5		1.4	100	13.0
Daughter Co. K.	1	F	W	17.5				11.0
Son N. K.	3	M	W	16.7		1.0	100	12.7
Son Go. K.	24	M	W	11.7		1.6	75	15.7
Wife P. K.	23	F	W	12.5		1.9	94	12.9
Son D. K.	2	M	W	19.5	15.0	1.9		11.8
Son M. K.	3	M	W	14.2	11.4	1.4		11.8
Daughter K. K.	6	F	W	15.3		1.2	100	12.4
Daughter Ki. K.	1	F	W	16.6	11.9	2.0	90	10.6
Daughter S. W.	29	F	W	14.7		1.6	100	16.3
Husband D. W.	33	M	W	13.5		1.5	100	18.0
Daughter D. W.	1	F	W	19.9		1.6	100	11.9
Daughter K. L.	28	F	W	11.3		1.5	91	13.9
Husband D. L.	33	M	W	13.1		1.3	88	15.9
Daughter V. L.	7	F	W	15.3		1.3	88	12.5
Son M. L.	5	M	W	13.6		1.4	90	12.0
Son L. L.	1	M	W	16.0		1.8		12.0

* Age at time of study.
† Propositus who had hemolytic anemia.
‡ History of sulfonamide induced hemolytic anemia 20 years ago.
§ Related by marriage.

TABLE V

Family studies of individuals with hemolytic anemia of unknown cause

Subject	Age*	Sex	Race	G-6-P.D.	6-PG.D.	Initial GSH	GSH protected	Hgb.
				ΔO.D./min./Gm. Hgb.		*mg./Gm. Hgb.*	*%*	*Gm./100 ml.*
Baby T.†	1 day	M	N	4.8	15.3	0.1	0	14.5
	2 days			4.5	15.4	1.2	16	14.7
	3 days			4.4	14.0	1.3	12	13.4
	6 days			3.1	13.6	1.4		12.7
Father T.	33 yrs.	M	N	10.7	12.8	1.8	75	15.4
Mother T.	31 yrs.	F	N	10.9	16.1	1.3	55	14.7
Sister T.	5 yrs.	F	N	10.5	13.1	1.5	63	12.2
Sister T.	3½ yrs.	F	N	14.5	14.5	1.4	92	12.9
H. W.†	8 yrs.	M	N	2.3	11.3	0.8	34	11.8
Father W.	40 yrs.	M	N	0.7	8.4	0.8	45	15.4
Brother W.	11 yrs.	M	N	1.5	11.2	0.5	45	12.0
Mother W.	38 yrs.	F	N	6.7	10.0	1.2	67	12.8

* Age at time of study.
† Propositus who had hemolytic anemia.

Glutathione stability

The control values for per cent of GSH protected after incubation with acetylphenylhydrazine are shown in Table I. Erythrocytes with a deficiency in G-6-P.D. were consistently found to have reduced GSH stability (Tables II through V). The preincubation concentrations of GSH were lower, on the average, in erythrocytes deficient in G-6-P.D. than in normal red blood cells. There was, however, a considerable overlap in GSH content between these two groups (Table VI).

The results of the GSH stability test expressed as "per cent of GSH protected" were compared to levels of G-6-P.D. (Figure 1). In subjects beyond the first 55 hours of life there was a significant positive correlation (correlation coefficient equals $\pm$ 0.79, p equals $<$ 0.001) between erythrocyte GSH stability and G-6-P.D. concentrations. This value was based on 123 subjects studied.

TABLE VI

Comparison of erythrocyte concentrations of reduced glutathione and glucose-6-phosphate dehydrogenase

Subject	No. of subjects	GSH Mean $\pm$ S.D.	G-6-P.D. Mean $\pm$ S.D.
		mg./Gm. Hgb.	*ΔO.D./Gm. Hgb./min.*
Normal enzyme			
Adults	76	1.47 $\pm$ 0.37	16.2 $\pm$ 2.2
Newborns	38	1.62 $\pm$ 0.26	20.4 $\pm$ 3.3
Deficient enzyme			
(all ages)	51	1.05 $\pm$ 0.39	4.7 $\pm$ 3.6

The transient instability of GSH in red blood cells of infants described by Zinkham and Childs (17) was confirmed for normal babies under 55 hours of age. The GSH remaining after incubation of erythrocytes of new borns with acetylphenylhydrazine was 17 to 50 per cent of the preincubation value compared to the normal adult range of 75 to 100 per cent (Table I).

6-Phosphogluconic dehydrogenase and purine nucleoside phosphorylase

The subjects with decreased levels of erythrocyte G-6-P.D. had concentrations of 6-PG.D. and PNP within the range of normal (Tables I through V).

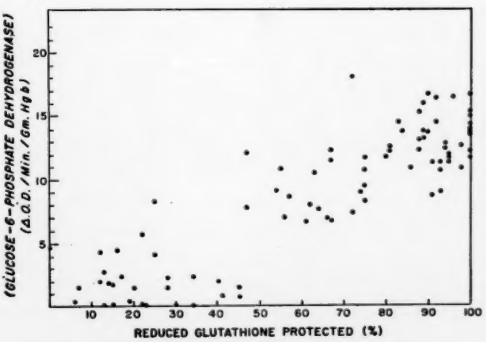

FIG. 1. THE RELATIONSHIP BETWEEN ERYTHROCYTE GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G-6-P.D.) CONCENTRATION AND REDUCED GLUTATHIONE (GSH) STABILITY UPON INCUBATION WITH ACETYLPHENYLHYDRAZINE

DISCUSSION

The hereditary basis for a deficiency of G-6-P.D. in human red blood cells has been demonstrated in the present study encompassing 18 families, each with at least two members possessing this enzymatic defect. The incidence of decreased erythrocyte G-6-P.D. activity among 103 relatives of 16 propositors was 38 per cent, as compared to an incidence of 4.6 per cent in the control population of 305 subjects (135 infants and 170 adults).[3] Of the 18 families studied, 15 were Negro families. Among the 152 Negro subjects in the control population, the incidence of this abnormality was 7.2 per cent. Among 153 Caucasian controls the incidence was 1.3 per cent.[4] The present findings as to the frequency of a deficiency in erythrocyte G-6-P.D. in a group of healthy subjects is in good agreement with earlier observations (5–7).

In 24 of the 52 subjects found to have a deficiency in erythrocyte G-6-P.D., the concentration of the enzyme appeared to be intermediate between the high levels of the controls and the low levels of those who gave a history of drug induced hemolytic anemias. Of these subjects with intermediate concentrations of enzyme, there was a striking predominance of females (23 out of 24 subjects). Females with either intermediate or low levels of erythrocyte G-6-P.D. had male offspring with low or normal, but not intermediate, levels, while among their female offspring, low, normal and intermediate levels occurred. In each of 12 families in which both parents could be tested, one parent had at least a moderate deficiency in red blood cell G-6-P.D. Male children were affected in 14 families, and in each instance the genetic defect was traced to the mother. Among 29 affected male subjects studied, only 4 were parents. The occurrence of the enzymatic defect mainly in male children and female parents, as well as the failure to trace the genetic defect in the male children to their fathers, suggest that sex linkage is the mode of inheritance. These findings are in accord with Childs and associates' (26) as well as Browne's (18) conclusions based on GSH stability studies. Childs has further suggested that the heterozygous and homozygous states may possibly be expressed in the intermediate and low levels of GSH stability (26). The present data, based on enzyme levels, suggest that such is not the case.[5] Although the size of this study does not permit a definite conclusion, the data best fit the postulate that the phenotypic expression of the gene is modified in the female.

Determination of the stability of erythrocyte GSH upon incubation with acetylphenylhydrazine yielded values that indicated a high degree of stability in erythrocytes with normal concentrations of G-6-P.D., markedly decreased stability in erythrocytes with moderate decrease in G-6-P.D. activity. This positive correlation between the level of erythrocyte G-6-P.D. and the stability of GSH is in accord with the previous evidence that erythrocyte GSH metabolism is related to the activity of G-6-P.D. (see reactions 1 through 4). The finding that an infant under three days of age is unable to protect GSH despite high levels of G-6-P.D. is unexplained. It has been shown (19) that the level of erythrocyte G-6-P.D. is very high at birth and does not decrease to within the range of adult levels for approximately 35 weeks. Glutathione reductase activity has been measured in erythrocytes of newborns and was found not to be diminished (20). Whatever the explanation, the fact that erythrocytes of newborn infants may have decreased GSH stability despite high levels of G-6-P.D. precludes the use of GSH studies as a screening test for hereditary erythrocyte drug sensitivity in young infants.

The identifications of G-6-P.D. as an important defect in drug sensitive erythrocytes may explain a curious clinical aspect of the drug induced hemolytic anemias, namely their tendency to be self-limited even in the face of continued ingestion of the agent (2). It has been demonstrated that older erythrocytes have an increased sensitivity to primaquine (21). Recent studies in normal persons (22, 23) have shown that the concentration of G-6-P.D. is high in young erythrocytes and decreases markedly with the *in vivo* aging of these cells. Similar studies in subjects with erythrocyte G-6-P.D. deficiency have revealed that the level

[4] Chi square test revealed that the incidence of erythrocyte G-6-P.D. deficiency in the control group and in the relatives of propositors differed significantly ($p \ll 0.001$).

[5] In one family a mother with a very low level of G-6-P.D. produced four normal sons. In another mating, a normal father and intermediate mother produced a daughter who was low.

of this enzyme, while not being comparable to normal values, is higher in young compared with old red cells (24). Thus, drug induced hemolysis may cease when the older cells, deficient in G-6-P.D., have been destroyed. The remaining red blood cell population would be composed of young cells, with higher levels of G-6-P.D., and hence, relatively resistant to hemolysis.

In this study the deficiency in G-6-P.D. was encountered in erythrocytes of individuals who never had hemolytic anemia as well as individuals with documented drug induced episodes of hemolysis.[6] It is not clear by what mechanism a deficiency in G-6-P.D. results in erythrocytes being sensitive to hemolysis following ingestion of certain drugs. Nevertheless, it would seem reasonable to conclude that individuals with a deficiency in erythrocyte G-6-P.D. may be expected to be susceptible to hemolysis following ingestion of such agents as naphthalene, primaquine, nitrofurantoin and fava beans. Accordingly, assay of erythrocyte G-6-P.D. may provide a ready means of detecting such individuals prior to their exposure to drugs potentially harmful to them.

SUMMARY

1. Erythrocyte glucose-6-phosphate dehydrogenase deficiency has been encountered in 4.6 per cent of an unselected population of 305 subjects. This enzymatic defect was more common among healthy Negroes (7.2 per cent) than among healthy Caucasians (1.3 per cent).

2. Erythrocyte glucose-6-phosphate dehydrogenase deficiency was detected in individuals with a history of hemolytic anemia due to naphthalene ingestion and fava bean consumption, and of undetermined cause.

3. A decrease in erythrocyte reduced glutathione stability upon incubation with acetylphenylhydrazine was consistently associated with a deficiency in glucose-6-phosphate dehydrogenase, except in very young infants. Infants under 55 hours of age demonstrated striking instability in reduced glutathione despite high levels of glucose-6-phosphate dehydrogenase activity.

4. The present data establish an hereditary basis for the deficiency of erythrocyte glucose-6-phosphate dehydrogenase. They strongly suggest that the gene is sex-linked and also sex-modified.

[6] During the preparation of this manuscript the finding of erythrocyte G-6-P.D. deficiency in erythrocytes of individuals who never had hemolytic anemia has also been reported by Schrier, Kellermeyer, Carson, and Alving (25) and is cited by Childs and co-workers (26).

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REFERENCES

1. Beutler, E., Dern, R. J., Flanagan, C. L., and Alving, A. S. The hemolytic effect of primaquine. VII. Biochemical studies of drug-sensitive erythrocytes. J. Lab. clin. Med. 1955, **45**, 286.
2. Dern, R. J., Beutler, E., and Alving, A. S. The hemolytic effect of primaquine. II. The natural course of the hemolytic anemia and the mechanism of its self-limited character. J. Lab. clin. Med. 1954, **44**, 171.
3. Zinkham, W. H., and Childs, B. Effect of naphthalene derivatives on glutathione metabolism of erythrocytes from patients with naphthalene hemolytic anemia (abstract). J. clin. Invest. 1957, **36**, 938.
4. Szeinberg, A., Sheba, C., Hirshorn, N., and Bodonyi, E. Studies on erythrocytes in cases with past history of favism and drug-induced acute hemolytic anemia. Blood 1957, **12**, 603.
5. Kimbro, E. I., Jr. The mechanism of hemolytic anemia induced by nitrofurantoin (Furadantin). Bull. Johns Hopk. Hosp. 1957, **101**, 118.
6. Beutler, E. The glutathione instability of drug-sensitive red cells. J. Lab. clin. Med. 1957, **49**, 84.
7. Hockwald, R. S., Arnold, J., Clayman, C. B., and Alving, A. S. Status of primaquine. 4. Toxicity of primaquine in Negroes. J. Amer. med. Ass. 1952, **149**, 1568.
8. Rall, T. W., and Lehninger, A. L. Glutathione reductase of animal tisues. J. biol. Chem. 1952, **194**, 119.
9. Marks, P. A. A newer pathway of carbohydrate metabolism; the pentose phosphate pathway. Diabetes 1956, **5**, 276.
10. Carson, P. E., Flanagan, C. L., Ickes, C. E., and Alving, A. S. Enzymatic deficiency in primaquine-sensitive erythrocytes. Science 1956, **124**, 484.
11. Waller, H. D., Löhr, G. W., and Tabatabai, M. Hämolyse und Fehlen von Glucose-6-phosphat-dehydrogenase in roten Blutzellen (Eine Fermen-

tanomalie des Erythrocyten). Klin. Wschr. 1957, **35**, 1022.

12. Marks, P. A. Red cell glucose-6-phosphate and 6 phosphogluconic dehydrogenases and nucleoside phosphorylase. Science, 1958, **127**, 1338.
13. Horecker, B. L., and Smyrniotis, P. Z. Phosphogluconic acid dehydrogenase from yeast. J. biol. Chem. 1951, **193**, 371.
14. Kornberg, A., and Horecker, B. L. Glucose-6-phosphate dehydrogenase *in* Methods in Enzymology, S. P. Colowick and N. O. Kaplan, Eds. New York, Academic Press Inc., 1955, vol. 1, p. 323.
15. Price, V. E., Otey, M. C., and Plesner, P. Preparation of nucleoside phosphorylase from calf spleen *in* Methods in Enzymology, S. P. Colowick and N. O. Kaplan, Eds. New York, Academic Press Inc., 1955, vol. 2, p. 448.
16. Grunert, R. R., and Phillips, P. H. A modification of the nitroprusside method of analysis for glutathione. Arch. Biochem. 1951, **30**, 217.
17. Zinkham, W. H., and Childs, B. Effect of vitamin K and naphthalene metabolites on glutathione metabolism of erythrocytes from normal newborns and patients with naphthalene hemolytic anemia. J. Dis. Child. 1957, **94**, 420.
18. Browne, E. A. The inheritance of an intrinsic abnormality of the red blood cell predisposing to drug induced hemolytic anemia. Bull. Johns Hopk. Hosp. 1957, **101**, 115.
19. Gross, R. T., and Hurwitz, R. E. A comparative study of the glycolytic and pentose phosphate pathways in red blood cells of newborns, prematures, infants, and children. J. Dis. Child. 1957, **94**, 487.
20. Gross, R. T., and Hurwitz, R. E. Unpublished data.
21. Beutler, E., Dern, R. J., and Alving, A. S. The hemolytic effect of primaquine. IV. The relationship of cell age to hemolysis. J. Lab. clin. Med. 1954, **44**, 439.
22. Marks, P. A. A relationship between human erythrocyte aging *in vivo* and the activities of glucose-6-phosphate and 6-phosphogluconic dehydrogenases (abstract). J. clin. Invest. 1957, **36**, 913.
23. Marks, P. A., Johnson, A. B., and Hirschberg, E. Effect of age on the enzyme activity in erythrocytes. Proc. nat. Acad. Sci. (Wash.). In press.
24. Gross, R. T., and Marks, P. A. Unpublished data.
25. Schrier, S., Kellermeyer, R., Carson, P., and Alving, A. S. A second enzyme abnormality in primaquine sensitive erythrocytes. J. Lab. clin. Med. 1957, **50**, 951.
26. Childs, B., Zinkham, W. H., Browne, E. A., Kimbro, E. L., and Torbert, J. V. A genetic study of a defect in glutathione metabolism of the erythrocyte. Bull. Johns Hopk. Hosp. 1958, **102**, 21.

STUDIES ON THE RELATIONSHIP BETWEEN GLUCOSE OXIDATION AND INTERMEDIARY METABOLISM. I. THE INFLUENCE OF GLYCOLYSIS ON THE SYNTHESIS OF CHOLESTEROL AND FATTY ACID IN NORMAL LIVER [1, 2]

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Since the original demonstration by Stetten and Boxer that the diabetic animal is relatively incapable of synthesizing fatty acids (2), numerous investigators have confirmed the marked dependence of fatty acid synthesis on glucose oxidation (3–5). Masoro, Chaikoff, Chernick, and Felts have shown that in the fasted as well as in the diabetic rat the production of fatty acids by the liver is sharply depressed (6). Reinstitution of carbohydrate oxidation by the administration of insulin to the diabetic animal or by the feeding of glucose alone to the fasted rat will readily restore their ability to synthesize fatty acids, while on the other hand, the administration of fat or protein at least to the latter animals has little or no such effect (6–9). These studies have strongly indicated that for "lipogenesis" [3] to proceed at an appreciable rate there exists a specific requirement for concomitant glucose breakdown. Furthermore, there is abundant evidence to demonstrate that lipogenesis is readily accelerated to above-normal levels by excessive glucose catabolism (6, 9, 10).

Cholesterol synthesis, too, appears to be in part controlled by the rate of glycolysis; however, this relationship is a more complicated one than that which exists between glycolysis and fatty acid synthesis. Tomkins and Chaikoff were the first to show that in the liver of the fasted rat cholesterol synthesis is greatly depressed (11). A specific dietary deficiency responsible for this effect was not established; however, it was demonstrated that the feeding of glucose alone was capable of restoring cholesterol synthesis to normal levels. In the diabetic animal cholesterol synthesis has been reported to be normal (4, 5) or at times decreased (4), and in yet another study cholesterolgenesis was found to be increased (12). The reason for these different results is not apparent, but it may be related to the relative severity of the diabetes and hence to the degree of impairment of glycolysis.

It is clear from these many lines of research, therefore, that the synthesis of both cholesterol and fatty acids is greatly influenced by the amount of glucose being oxidized within the cell; however, the mechanism by which glucose produces these effects has remained obscure.

It has been recently demonstrated that, contrary to older concepts, glucose can be broken down in

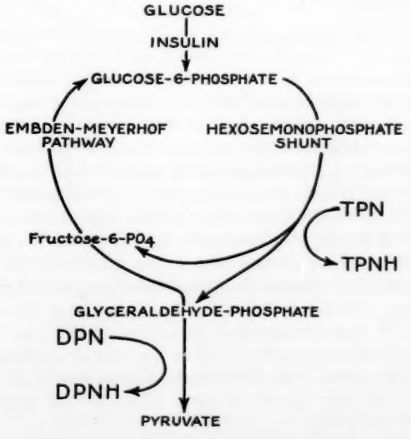

FIG. 1. SUMMARY OF THE ROUTES OF HEPATIC GLYCOLYSIS

[1] A preliminary report of these studies has been previously published (1).

[2] These studies were supported by grants from the American Heart Association, the Dallas Heart Association, and the National Heart Institute, United States Public Health Service.

[3] As is customary, "lipogenesis" is used in this and the following paper to refer to the synthesis of fatty acids; "lipid" will here refer to the two lipids under study, namely cholesterol and fatty acids.

the liver by either of two pathways (Figure 1). One is the well known Embden-Meyerhof (EM) route over which most of the glucose of the liver cell is probably oxidized; the other is the hexosemonophosphate (HMP) shunt. The quantitative importance of HMP glycolysis [4] is still uncertain. The results of some studies have indicated that this pathway may account for as much as 30 to 50 per cent of hepatic glycolysis (13–15), while in other experiments the HMP route has apparently been responsible for less than 10 per cent of the glucose metabolized by the liver (14, 16–18). In spite of the fact that the HMP shunt may play a quantitatively minor role in glucose oxidation, it seemed possible that this pathway might exert an influence on the regulation of lipid synthesis out of proportion to its function in glucose metabolism. For this reason a study of the relationship between glycolysis and the synthesis of fatty acids and cholesterol has been carried out using cell-free preparations of rat liver. The relative importance of each of the two routes of glucose breakdown in the control of lipid synthesis has been evaluated by using acetate-1-C^{14} to determine the rates of synthesis while glucose oxidation was stimulated alternately via the Embden-Meyerhof pathway or via the hexosemonophosphate shunt. Studies of the influence of glycolysis on lipid synthesis in normal liver are reported here. An investigation of the cause of the lipogenic defect in diabetes is described in the following paper (19).

EXPERIMENTAL PROCEDURE

Use was made in these studies of carefully homogenized, cell-free preparations of rat liver. As prepared, such systems have the advantage that they will carry out most of the reactions of the normal cell, and yet their use allows one to manipulate directly many of these metabolic processes. These preparations thus provide easily controlled models from which inferences can be drawn regarding metabolic processes in the intact cell.

The method used to investigate the function of the two pathways of glucose oxidation was suggested by the finding of Wenner, Dunn, and Weinhouse, that the addition of diphosphopyridine nucleotide (DPN) to liver homogenates markedly increases the rate of oxidation of glucose (20). This DPN-stimulated breakdown of glucose was later shown to occur primarily via Embden-Meyerhof glycolysis (14). These authors have also reported a single experiment in which supplementation with both triphosphopyridine nucleotide (TPN) and DPN caused an increase in glucose oxidation which occurred predominantly by way of the HMP route (14).

Studies in our laboratory have confirmed and extended these observations (21). We have demonstrated that the addition of TPN alone to a liver homogenate markedly stimulates the oxidation of glucose over the hexosemonophosphate pathway. DPN alone, as was also indicated by Wenner's studies, enhances primarily EM glycolysis.

A means is therefore available for stimulating glucose breakdown primarily down one glycolytic pathway or the other (Figure 1). By supplementing a homogenate with either DPN or TPN and simultaneously measuring the rates of incorporation of acetate-1-C^{14} into both cholesterol and fatty acids, the role of each of these two pathways of glucose oxidation in controlling lipid synthesis could be studied.

Treatment of animals. Male, Long-Evans rats obtained from the Diablo Animal Laboratories, Berkeley, Calif., were used throughout this study. Their weights ranged from 150 to 250 Gm. They were maintained on Purina Laboratory Chow except for a two or three day period prior to killing when they were given *ad libitum* a 67 per cent glucose diet.[5] Only rats that had food in their stomachs at the time of death were used in the experiments.

Preparation of homogenates and incubation procedure. Animals were stunned by a blow on the head, their throats cut, and their livers perfused through the portal vein with 15 ml. of the ice-cold phosphate buffer, 0.1 M, pH 7.7. The livers were then quickly excised, placed in the cold buffer and allowed to cool. In early studies ice buckets were used for this purpose; however, more uniform results were obtained if the entire procedure was carried out in a room maintained at 5° C. The livers were weighed, placed in a Dounce homogenizer (22) and a volume of phosphate buffer equal to the weight of the liver, plus glutathione [6] to make a final concentration of 10 μM per ml. were added. The livers were then homogenized with five to six slow strokes of a plunger having a clearance of about 0.5 to 1 mm. This is looser than the "loose" plunger supplied with the commercial Dounce homogenizer. The resulting homogenate was centrifuged at 800 × G for 10 minutes at −1° C., and the supernatant containing the broken liver cells minus nuclei was used as described below.

[4] "Glycolysis" will be used in this paper to indicate the breakdown of glucose by either the Embden-Meyerhof pathway or the hexosemonophosphate shunt.

[5] The composition of the diet was as follows: glucose, 67 per cent; casein, 25 per cent; Hawk-Oser salt mixture, 7 per cent; vitamins per 1,000 Gm. of diet—inositol, 500 mg.; folic acid, 10 mg.; 0.1 ml. oleum percomorphum containing vitamin A, 6,000 units and vitamin D, 850 units; one bottle of Folbesyn® (Lederle) containing thiamine, 10 mg., riboflavin, 10 mg., sodium pantothenate, 10 mg., niacinamide, 50 mg., pyridoxine, 5 mg., B-12, 15 μg., and ascorbic acid, 300 mg.

[6] The omission of glutathione was found to have no detectable influence on lipogenesis.

TABLE I

Effect of homogenate dilution on lipid synthesis *

Ratio of liver weight to buffer volume	Per cent of added acetate-1-C^{14} recovered in:		
	Fatty acids	Cholesterol	CO_2
1 : 3.0	1.1	0.02	0.6
1 : 2.0	6.6	0.17	1.3
1 : 1.5	14.3	0.34	3.0
1 : 1.25	20.5	0.36	3.8
1 : 1.0	35.3	0.63	8.3

* Concentration of additions: glucose-6-phosphate, 18×10^{-3} M; TPN, 0.7×10^{-3} M; potassium acetate, 2×10^{-3} M in a total volume of 1.35 ml. (Incubated for one hour at 37° C.)

Such a homogenate represents a relatively concentrated preparation of liver tissue. It can be readily centrifuged and pipetted and provides a minimum dilution of the soluble enzymes and cofactors of the cell. This preparation will synthesize fatty acids and with added coenzymes will also synthesize cholesterol; it will often perform these functions at rates comparable to those seen in liver slices (Table I). More dilute homogenates, as also shown in Table I, were found to have less ability to carry out these processes and their use was abandoned.

All incubations were carried out in the modified Warburg flasks designed by Baruch and Chaikoff (23). Sealing such vessels with serum caps allows samples to be withdrawn or additions to be made without opening the flasks. Either 1 or 2 ml. of the homogenate was placed in the outer chamber of the flask and the substrates,[7] coenzymes,[8] acetate-1-C^{14} [9] and water were then added as indicated in Table II to give a constant volume of either 1.4 or 2.5 ml. The flasks were next gassed with 95 per cent O_2, 5 per cent CO_2 for 10 seconds and incubated at 37.5° C. in a Dubnoff shaker for one hour unless otherwise indicated.

ANALYTICAL METHODS

$C^{14}O_2$. At the end of the incubation the flasks were placed in an ice bath and 1 ml. of alkaline Hyamine 10X 1:1 methanol-toluene, prepared as described by Passmann, Radin, and Cooper (24), was injected through the serum cap into the center well. Two-tenths ml. of 10 N sulfuric acid was injected into the outer well containing the incubation mixture; the flasks were then shaken at 0 to 5° C. for 30 minutes to allow the Hyamine to absorb the $C^{14}O_2$. It was found necessary to carry out this step at reduced temperature in order to prevent acetic acid-C^{14} from being absorbed by the Hyamine solution. Trapping the CO_2 at a temperature of 37° C. resulted in as much as 1 per cent of the acetate-C^{14} appearing in the Hyamine in the 30 minute period.

The Hyamine-$C^{14}O_2$ solution was next transferred quantitatively with toluene into a 10 ml. volumetric flask. A 2 ml. aliquot was placed in 16 ml. of a 0.4 per cent solution of diphenyloxazole in toluene and the C^{14} assayed in a Packard Liquid Scintillation Counter.[10] Total CO_2 was determined with the use of a Van Slyke manometric apparatus.

Cholesterol-C^{14} determination. The homogenate was saponified by adding 90 per cent (w/v) potassium hydroxide (1 ml. to 2.5 ml. of incubation mixture or 0.5 ml. to the 1.4 ml. mixture) and heating in an autoclave at 15 pounds pressure for one hour. The saponified homogenate was then transferred to a 250 ml. Erlenmeyer flask, and ethanol was added to give a 50 per cent solution. The nonsaponifiable material was next extracted with petroleum ether and the cholesterol fraction (which includes other β sterols) was isolated as the digitonide by the procedure of Sperry and Webb (25). The digitonide was dissolved with heating in 2.2 ml. of methanol and 1 ml. was added to 16 ml. of the liquid scintillation solution for C^{14} determination.

Fatty acid-C^{14} determination. Method I: After extraction of the cholesterol the saponified homogenate was acidified, and the fatty acids were isolated by extensive extraction of the mixture with petroleum ether. The pooled extracts were concentrated, washed twice with water and made up to a 10 ml. volume. A 2 ml. aliquot of the petroleum ether extract was assayed for fatty acid-C^{14} as described above.

Method II: In later studies an improved rapid method for fatty acid isolation was devised. This gave clear solutions which did not cause the slight quenching of the liquid scintillator which the somewhat yellow solutions of Method I occasionally produced. After removing the nonsaponifiable material as described above, the 250 ml. Erlenmeyer flask was heated on a steam bath to remove the ethanol. Water was then added to give a volume of about 5 to 6 ml. and the mixture was acidified below pH 3.0 with concentrated sulfuric acid. After cooling, exactly 20 ml. of petroleum ether was added; the flask was then stoppered and shaken for 15 minutes at top speed in an International Bottle Shaker, Size 2. Approximately 5 ml. of the clear supernatant was placed in a 10 ml. glass-stoppered centrifuge tube and washed twice with 5 ml. portions of water. A 2 ml. aliquot of the petroleum ether was then taken for assay of the fatty acid-C^{14}.

RESULTS

Effect of glycolytic pathway on fatty acid synthesis

The results of stimulation of the Embden-Meyerhof or the hexosemonophosphate pathways

[7] Glucose-6-phosphate was obtained from the Sigma Chemical Company; isocitrate was purchased from Nutritional Biochemical Inc.

[8] Triphosphopyridine nucleotide and diphosphopyridine nucleotide were purchased either from the Sigma Chemical Company or the Pabst Laboratories.

[9] Acetate-1-C^{14} was prepared by Tracerlab, Inc., or the New England Nuclear Corporation.

[10] Packard Instrument Co., LaGrange, Ill.

TABLE II

*Influence of glycolytic pathways upon lipid synthesis in normal livers**

Experiment	Glycolytic pathway stimulated	Acetate-1-C^{14} converted to: Fatty acid	Cholesterol	CO_2
		mμM	*mμM* $\times 10^{-2}$	*mμM*
1	Neither	7	<6	297
	HMP	46	374	564
	EM	20	<6	114
	HMP+EM	304	172	536
2	Neither	17	<11	415
	HMP	760	440	376
	EM	81	<11	381
	HMP+EM	2,057	229	246
3	Neither	6	<22	336
	HMP	1,254	2,982	193
	EM	77	<22	161
	HMP+EM	3,213	1,099	131
4	Neither	2	<6	11
	HMP	70	1,580	64
	EM	13	<6	9
	HMP+EM	775	686	54
5	Neither	10	<5	257
	HMP	723	228	60
	EM	52	<5	252
	HMP+EM	738	81	99
6	Neither	4	<5	194
	HMP	173	3,279	154
	EM	17	<5	210
	HMP+EM	178	1,397	124
7	Neither	0.3	<2	74
	HMP	85	179	111
	EM	2	<2	130
	HMP+EM	75	66	114
8	Neither	1	10	37
	HMP	113	867	100
	EM	8	10	96
	HMP+EM	85	362	69
9	Neither	0.6	36	14
	HMP	43	6,819	142
	EM	2	20	49
	HMP+EM	43	3,575	111
10	Neither	4	5	179
	HMP	196	3,881	144
	EM	32	15	134
	HMP+EM	147	1,392	100
11	Neither	0.7	<3	22
	HMP	361	452	112
	EM	9	10	135
	HMP+EM	255	99	84
12	Neither	0.5	<5	17
	HMP	39	61	116
	EM	3	<5	101
	HMP+EM	79	46	118
13	Neither	0.8	15	3
	HMP	49	51	20
	EM	2	20	11
	HMP+EM	128	36	36
14	Neither	1	20	6
	HMP	10	76	33
	EM	4	<5	79
	HMP+EM	41	51	34
15	Neither	1	<5	61
	HMP	19	50	60
	EM	3	8	64
	HMP+EM	34	485	63
16	Neither	1	20	15
	HMP	94	161	54
	EM	3	20	19
	HMP+EM	65	56	41
17	Neither	0.8	15	4
	HMP	12	15	46
	EM	2	10	15
	HMP+EM	17	10	66
18	Neither	0.3	15	3
	HMP	30	87	98
	EM	9	15	18
	HMP+EM	20	66	
19	Neither	4	1	16
	HMP	31	158	164
	EM	5	16	83
	HMP+EM	46	91	150
20	Neither	2	10	15
	HMP	155	6,222	96
	EM	42	31	346
	HMP+EM	167	2,372	306
21	Neither	4	<5	276
	HMP	524		230
	EM	0.8	<5	257
	HMP+EM	606	1,326	207
22	Neither	5	<5	454
	HMP	112	9,614	261
	EM	3	31	357
	HMP+EM	260	8,787	321
23	Neither	2	<5	98
	HMP	339	7,211	347
	EM	16	15	363
	HMP+EM	275	3,942	189
24	Neither	1	5	174
	HMP	111	676	140
	EM	37	10	194
	HMP+EM	173	343	94
25	Neither	2	0.5	235
	HMP	583	185	121
	EM	19	3	216
	HMP+EM	412	49	100
26	Neither	62	<5	853
	HMP	447	1,452	187
	EM	26	<5	910
	HMP+EM	447	716	359
27	Neither	5	<3	270
	HMP	235	2,704	174
	EM	5	5	276
	HMP+EM	372	1,565	158
28	Neither	3	<3	192
	HMP	242	55	113
	EM	30	<3	146
	HMP+EM	262	13	90
29	Neither	4	<3	186
	HMP	231	1,097	163
	EM	16	10	192
	HMP+EM	247	486	89
30	Neither	0.2	<5	80
	HMP	10	82	78
	EM	0.9	<5	87
	HMP+EM	17	240	86

* Experiments 1 through 23 contained glucose-6-phosphate, 20×10^{-3} M; TPN, 0.8×10^{-3} M; DPN, 0.8×10^{-3} M. The potassium acetate concentration in Experiments 3 and 15 was 4×10^{-3} M; in all others, it was 2×10^{-3} M. Total volume was 2.5 ml.

Experiments 24 through 30 contained glucose-6-phosphate, 18×10^{-3} M; TPN, 0.7×10^{-3} M; DPN, 0.7×10^{-3} M; potassium acetate, 2×10^{-3} M. Total volume was 1.4 ml.

by the addition of DPN or TPN, respectively, are shown in Table II. Glucose-6-phosphate was used as the glycolytic substrate in this study since it represents the first metabolite of glucose that is common to both pathways of glucose oxidation. As may be seen, the synthesis of fatty acids can be carried out in this system at small but significant rates even in the absence of added coenzymes. Stimulation of the Embden-Meyerhof pathway caused a moderate change in fatty acid synthesis which varied from a fivefold depression to a thirty-seven fold increase. On the other hand, enhancement of the HMP route consistently produced a very striking increase in the synthesis of fatty acids which amounted to at least 2 to 47 times that *produced by EM stimulation.* It is apparent, therefore, that in some manner stimulation of glucose oxidation via the HMP shunt has a far greater effect upon lipogenesis than does glycolysis via the EM route. Stimulation of both pathways of glucose oxidation usually produced an even greater accentuation of lipogenesis than that caused by the stimulation of either pathway alone; however, in about one-third of the experiments this effect was not observed.

Effect of glycolysis on cholesterol synthesis

A similar dependence of cholesterol synthesis on glucose oxidized over the HMP pathway is demonstrated in Table II. In homogenates, unsupplemented with cofactors, the synthesis of cholesterol often did not proceed at a detectable rate. Stimulation of the EM pathway had little or no effect on this process; however, enhancement of glucose oxidation via the HMP shunt caused a very marked increase in cholesterolgenesis in all but 1 of the 30 experiments. The process of cholesterol synthesis as well as that of fatty acid synthesis would therefore appear to be dependent upon the glucose using the TPN-dependent route of glucose oxidation.

It should be noted, however, that in all except Experiments 15 and 30, the addition of both TPN and DPN to the system caused a consistent depression of cholesterolgenesis relative to that seen when only the HMP shunt was stimulated (Table II). This finding is in contrast to the case of fatty acid synthesis where the presence of both cofactors usually produced maximal lipogenesis.

Carbon dioxide-C^{14} production

The production of $C^{14}O_2$ by the homogenate is no doubt an indication of the functioning of the Krebs cycle in this system. The amount of $C^{14}O_2$ released varied considerably from one experiment to another; however, the influence of glycolysis upon fatty acid and cholesterol synthesis described above was consistently observed despite these variations. It should be noted that frequently (14 out of 30 experiments) the amount of $C^{14}O_2$ produced was not significantly increased by the addition of either or both coenzymes and in the experiments recorded in Table III, in which total $C^{12}O_2$ plus $C^{14}O_2$ production was also measured, neither the total CO_2 nor the specific activity of the $C^{14}O_2$ was stimulated by DPN or TPN.

TABLE III

Influence of triphosphopyridine nucleotide (TPN) and diphosphopyridine nucleotide (DPN) on CO_2 production in normal liver *

Experiment	Coenzyme	$C^{14}O_2$ (% of added acetate-1-C^{14})	Total CO_2 ($C^{12}O_2+C^{14}O_2$)	Specific activity $\left[\frac{C^{14}O_2 \text{ (\% of acetate-}C^{14})}{\text{Total } CO_2 \ (m\mu M)}\right]$
			μM	
1	None	13.9	244	57.0
	TPN	8.3	231	35.9
	DPN	9.6	246	39.0
	TPN+DPN	7.5	222	33.8
2	None	8.8	232	37.9
	TPN	3.6	228	15.8
	DPN	7.1	222	32.0
	TPN+DPN	3.5	237	14.8

* Concentration of additions: glucose-6-phosphate, 18×10^{-3} M; TPN, 0.7×10^{-3} M; DPN, 0.7×10^{-3} M; potassium acetate, 2×10^{-3} M in a total volume of 1.35 ml.

Rate studies of lipid synthesis

The previous experiments employed incubations carried out for a single period of time, namely 60 minutes. In order to determine whether the conclusions drawn from these single determinations were valid, a study of the rates of incorporation of acetate-1-C^{14} into fatty acids and cholesterol was carried out by measuring the extent of synthesis of cholesterol-C^{14} and fatty acid-C^{14} at intervals of from 0 to 90 minutes.

The slopes of curves obtained by plotting such data on linear coordinates represent the rates of synthesis of lipid-C^{14}. As can be observed in Figure 2, at all time intervals studied, EM glycolysis exerted but little influence upon the synthesis of fatty acids. On the other hand, as indicated by the slopes of these curves, stimulation of the HMP pathway caused a marked increase in the rate of lipogenesis, and the addition of both DPN and TPN increased this rate still further. Inasmuch as the synthesis of fatty acids followed a linear curve from 15 to approximately 60 minutes, a 60 minute period of incubation was routinely used in these studies.

The rate of synthesis of cholesterol after a 15 minute period was also observed to be approximately constant up to a period of 60 minutes and again the results of such rate studies confirm those of the previous section (Figure 3). Enhancement of the EM pathway at no time interval produced a significant increase in the rate of cholesterol-C^{14} synthesis; yet acceleration of the HMP pathway greatly increased this synthesis. Again the simultaneous stimulation of both pathways of glucose breakdown caused a *decrease* in the slope of the curve of cholesterol-C^{14} production compared to that observed when only the HMP pathway was stimulated.

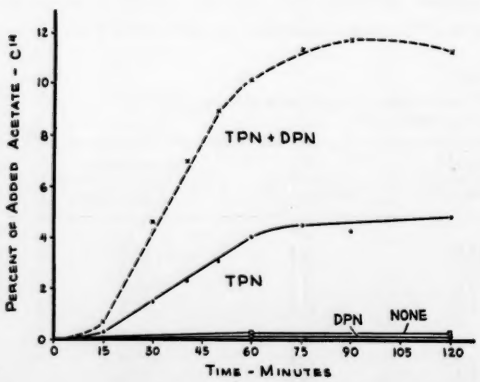

FIG. 2. THE RATES OF SYNTHESIS OF FATTY-ACID-C^{14}. Conditions were as described for Experiment 1, Table II.

Mechanism of HMP stimulation of fatty acid and cholesterol synthesis

The preceding experiments demonstrate that stimulation of the HMP shunt markedly accelerates the processes of lipogenesis and cholesterolgenesis; however, these studies leave unanswered the question as to how this pathway of glycolysis produces these striking effects.

As can be observed in Figure 1, glucose oxidized via the HMP shunt causes the production of reduced TPN (TPNH) and if the glyceraldehyde phosphate or fructose phosphate produced is converted to pyruvate, DPNH is formed as well. In contrast, glucose oxidized via the EM pathway yields only DPNH. Since TPNH is now known to be required in the synthesis of fatty acids (26) and, as we have previously suggested (27), is probably specifically required in cholesterol synthesis as well, it seemed likely that it is this cofactor, produced only by the HMP route, which mediates the effects of HMP glycolysis.

To test this possibility an alternate TPNH generating system was employed. Isocitrate and TPN will also yield TPNH, and as shown in Table IV the addition of this generating system will cause a very substantial increase in both fatty acid and cholesterol synthesis. It is therefore likely that the ability of HMP glycolysis to stimulate lipid synthesis is indeed due to the TPNH generated during glucose oxidation via this pathway. Neither isocitrate alone nor TPN alone will stimulate the synthesis of fatty acids as markedly as will both together. Cholesterol synthesis, however, was definitely stimulated by TPN in the absence of added substrate. This last result is probably due to a breakdown of endogenous glucose sufficient in the presence of supplemental TPN to yield TPNH in quantities large enough to cause the stimulation of cholesterol synthesis. The lack of a similar response in fatty acid synthesis may be explained on the basis of a greater affinity of the

TABLE IV

Influence of reduced triphosphopyridine nucleotide (TPNH) generating systems upon fatty acid and cholesterol synthesis *

Experiment	Cofactor and/or substrate	Acetate-C^{14} converted to: Fatty acids	Cholesterol
		$m\mu M$	$m\mu M \times 10^{-2}$
1	None	2	10
	Glucose-6-phosphate	4	5
	Potassium isocitrate	9	10
	TPN+glucose-6-phosphate	133	960
	TPN+potassium isocitrate	314	550
	TPN	17	190
2	Glucose-6-phosphate	2	5
	Sodium isocitrate	3	10
	TPN+glucose-6-phosphate	96	764
	TPN+sodium isocitrate	370	114
	TPN	22	307

* Concentration of additions: TPN, 0.8×10^{-3} M; glucose-6-phosphate, 21×10^{-3} M; isocitrate, 21×10^{-3} M in a total volume of 2.7 ml.

cholesterol synthesizing enzymes for the available TPNH.

It is also noteworthy that in the presence of TPN the use of glucose-6-phosphate as a substrate yielded greater amounts of cholesterol-C^{14} than did isocitrate. The reverse, however, was true for fatty acid synthesis; in this case isocitrate plus TPN caused a greater stimulation than did glucose-6-phosphate plus TPN.

DISCUSSION

The function of glycolysis in the economy of cellular metabolism is at present only incompletely understood. It has frequently been assumed that glucose catabolism serves mainly to supply energy in the form of ATP during the glycolytic process following which the remaining carbon substrates may be used for synthetic purposes and for the energy yielding reactions of aerobic oxidation. Evidence is now accumulating to indicate that glucose actually represents a relatively minor energy source for many tissues of the body (28, 29). Furthermore, the fact that only small amounts of glucose are required to produce rather profound effects in intermediary metabolism is not consistent with the concept that glycolysis acts simply as a source of nonspecific energy or of two-carbon units.

A different function for glycolysis in intermediary metabolism is suggested by the findings

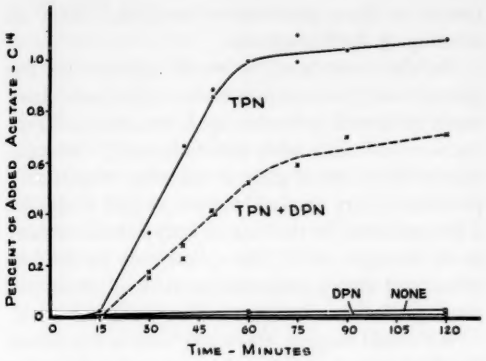

FIG. 3. THE RATES OF SYNTHESIS OF CHOLESTEROL-C^{14}

Conditions were the same as those for Experiment 1, Table II.

reported here; namely, that this phase of glucose oxidation functions at least in part as a generating system for reduced coenzymes, which by donating their protons to specific substrates in turn control various synthetic processes of the cell. In this manner the rate of glucose oxidation may serve to regulate the rates at which these various synthetic reactions occur. That such a mechanism is in part dependent upon the *route* by which glucose is oxidized is also suggested by the results of this study.

The specific reactions examined in the present investigation were those leading to the synthesis of fatty acids and of cholesterol. By stimulating glucose oxidation via either of the two pathways of glucose catabolism, *i.e.*, the Embden-Meyerhof route and the hexosemonophosphate shunt, it was possible to examine the part played by each of the glycolytic pathways in regulating the synthesis of these lipids.

The rather surprising observation was made that in spite of its major role in the glycolytic process the EM pathway appears to be relatively unimportant in controlling lipid synthesis. It would follow, therefore, that the cofactors produced during the operation of this pathway, *i.e.*, DPNH and ATP, are not limiting in the synthesis of these two lipids. In other words, though these cofactors may be required for lipid synthesis (26, 30, 31), they are probably supplied in sufficient quantity by the normally rapid Embden-Meyerhof glycolysis of the liver cell so that a further in-

crease in their production has little effect in stimulating lipid synthesis.

On the other hand, it would appear that the glucose which uses the hexosemonophosphate shunt has a profound influence upon the rates of synthesis of both fatty acids and cholesterol. Stimulation of this route of glucose oxidation consistently produced a very marked increase in lipid synthesis. This amounted in the case of fatty acid formation to an average of 102 times that seen in the unstimulated homogenate and in the case of cholesterol synthesis to an average increase of 293-fold.

We would suggest, therefore, that in conditions in which active glycolysis causes a stimulation of fatty acid and cholesterol synthesis this enhancement of lipid synthesis is due primarily to the relatively small portion of glucose which is oxidized via the hexosemonophosphate shunt.

These results are no doubt independent of the operation of the Krebs cycle and the TPNH thus produced since, in about one-half of the experiments shown in Table II, supplementing the system with either DPN or TPN caused no significant increase in the amount of $C^{14}O_2$ produced from the acetate-C^{14}; nonetheless, fatty acid and cholesterol synthesis still responded as described. These findings would also indicate that the pyridine nucleotides are often present within the cell in concentrations sufficient for the reactions of the citric acid cycle to proceed at optimal rates. It is also of interest that from evidence presented in Table III, the total amount of CO_2 produced ($C^{12}O_2$ plus $C^{14}O_2$) need not be increased by the addition of the cofactors. In view of the fact that the oxidation of glucose to CO_2 is stimulated in liver homogenates by the addition of either DPN or TPN (21), other sources of Krebs cycle intermediates must under these circumstances decrease their contribution to this cycle, thereby maintaining the total CO_2 production constant.

The factor mediating the effects of glucose oxidation upon both fatty acid and cholesterol synthesis is in all probability the reduced TPN produced during the functioning of the HMP pathway, since the substitution of another TPNH generating system, isocitrate and TPN, readily replaced the hexosemonophosphate shunt in enhancing both of these syntheses (Table IV).

The probable sites of action of the reduced pyridine nucleotides in lipid synthesis are shown in Figure 4. Langdon has demonstrated that TPNH is required in the synthesis of fatty acids and that the reaction requiring this cofactor is the one involving the conversion of crotonyl-CoA to butyryl-CoA (26). Seubert, Greull, and Lynen (32) have reconstructed a fatty acid synthesizing system from liver which also requires TPNH. Brady, Mamoon, and Stadtman have shown that in the supernatant fraction of pigeon liver TPNH is a limiting cofactor for fatty acid synthesis (33); and since completion of our study, Langdon has made the same observation in normal rat liver homogenates (34). Dituri, Shaw, Warms, and Gurin, in studying liver mitochondria (35), and Hele, Popjak, and Lauryssens, using mammary gland (36), have not been able to confirm these findings and present evidence to indicate that DPNH is the limiting factor in fatty acid synthe-

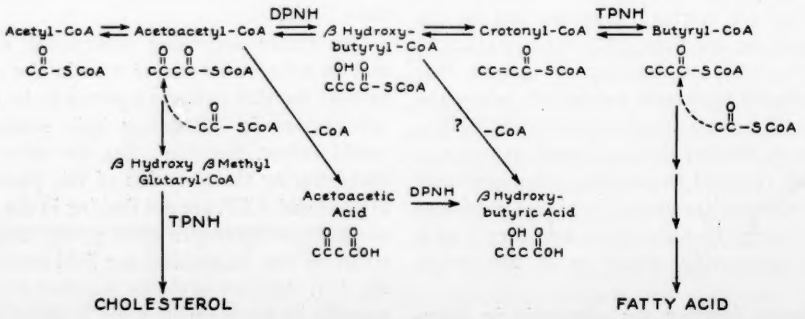

FIG. 4. DETAILS OF THE RELATIONSHIP BETWEEN CHOLESTEROL AND FATTY ACID SYNTHESIS

sis. Our results are clearly more in accord with those of Brady, Mamoon, and Stadtman and of Langdon.

The effects on lipid synthesis of simultaneous stimulation of both pathways of glycolysis are of particular interest. The addition of both TPN and DPN to the liver system was usually found to increase the rate of fatty acid synthesis above that produced by TPN alone, and it may therefore be concluded that when the HMP shunt is functioning rapidly, the rate of Embden-Meyerhof glycolysis may become a limiting factor in fatty acid synthesis. It should be noted, however, that the HMP shunt joins the EM pathway at the glyceraldehyde phosphate and fructose phosphate levels (Figure 1) and the conversion of these latter compounds to pyruvate then requires the action of DPN. It is likely, therefore, that the presence of both pyridine cofactors causes an even greater stimulation of the HMP shunt than does TPN alone. This conclusion is supported by the fact that in studies to be reported elsewhere a maximal oxidation of glucose-1-C^{14} was obtained under the combined influence of TPN and DPN (21).

In contrast to the observed increase in fatty acid synthesis, the stimulation of both pathways of glucose oxidation produced a consistent *depression* in the synthesis of cholesterol relative to that observed when only the HMP shunt was stimulated. This would suggest that in the presence of adequate glycolysis cholesterol synthesis within the cell might be regulated by the relative amounts of glucose which proceed via each of the pathways of glucose oxidation: That glucose using the HMP route would stimulate cholesterol synthesis while that traversing the EM pathway would depress this process. A possible explanation for the depressant effect of EM glycolysis upon cholesterol synthesis may be found by examining the metabolic pathways of fatty acid and cholesterol synthesis as shown in Figure 4.

The synthesis of both fatty acids and cholesterol begins with the condensation of two molecules of acetyl-coenzyme A (acetyl-CoA) to form one of acetoacetyl-CoA. This compound can then lose its CoA to form free acetoacetic acid or can be reduced by DPNH to yield β-hydroxybutyryl-CoA. By the removal of water β-hydroxybutyryl-CoA becomes crotonyl-CoA which is then reduced by TPNH to give the four-carbon, activated fatty acid, butyryl-CoA. This compound can be attacked at its fourth carbon by another molecule of acetyl-CoA; the resulting six carbon molecule can then by a series of reductions and condensations become a long chain fatty acid (37, 38).

If, on the other hand, the four-carbon compound attacked by the acetyl-CoA is *acetoacetyl-CoA* rather than butyryl-CoA, the molecule formed is β-hydroxy-β-methylglutaric acid (or its CoA derivative) (39), and this acid is believed to lead to the synthesis of cholesterol (40). The evidence presented here would indicate that there is a specific requirement for TPNH in the process of cholesterol synthesis. In view of this finding the requirement for DPN and ATP in the synthesis of cholesterol noted by Bucher (41) may be due to the known conversion of these two cofactors to TPN. A recent report of Tchen and Bloch (31) has indicated that the site of action of TPNH in cholesterol synthesis is at the cyclization of squalene to yield the sterol nucleus; however, TPNH did not appear to be a specific requirement since DPNH was also capable of performing this function.

In our study TPNH would clearly seem to be the specific factor which stimulates the synthesis of cholesterol. The relative depressant effect of DPN might then be explained as being secondary to the increase in fatty acid synthesis produced by the addition of DPN as well as TPN to the system. Such an increase in fatty acid synthesis may remove either acetoacetyl-CoA or TPNH to an extent that these compounds are no longer present in amounts capable of maintaining a rapid rate of cholesterol synthesis. The fact that EM glycolysis would appear to reduce cholesterol synthesis suggests than an *in vivo* method for directing glucose down this pathway at the expense of the HMP shunt might provide a means for controlling excessive cholesterol synthesis.

Finally, it is of interest that the use of *isocitrate* and TPN as an alternate generating system of TPNH caused an even greater enhancement of fatty acid synthesis than did glucose-6-phosphate and TPN (Table IV). On the other hand, glucose-6-phosphate proved to be a better substrate for cholesterol synthesis than did isocitrate. Since presumably both cholesterol and fatty acids are synthesized exclusively from acetyl-CoA (Figure

4), these differences in the stimulation of lipid synthesis cannot be explained on the basis of differential dilution of separate precursor pools. One explanation for these results is that in some manner isocitrate may donate its protons via TPNH more specifically to the precursors of fatty acids while glucose-6-phosphate may more readily contribute its protons, also via TPNH, to cholesterol. This conclusion is analagous to that previously made by Hoberman based on the finding that protons from lactate are incorporated preferentially into glycogen rather than into glutamate (42).

SUMMARY

The role of glucose oxidation in the regulation of cholesterol and fatty acid synthesis has been studied in a cell-free preparation of normal rat liver. It is concluded that the stimulatory effect of glycolysis upon these syntheses is due primarily to that portion of glucose oxidation which proceeds via the hexosemonophosphate (HMP) shunt. Evidence is presented to demonstrate that it is the reduced triphosphopyridine nucleotide (TPNH) generated by the hexosemonophosphate pathway which mediates this effect of glycolysis upon both fatty acid and cholesterol synthesis. When glucose is being oxidized at a rapid rate, it would appear that the synthesis of cholesterol may be regulated by the amount of glucose using each glycolytic pathway, oxidation via the HMP route stimulating and that via the Embden-Meyerhof pathway depressing sterol synthesis.

Finally, it is suggested that the process of glucose catabolism, by donating protons to specific sites in intermediary metabolism, may serve the primary function of regulating certain synthetic processes of the cell.

REFERENCES

1. Siperstein, M. D., and Fagan, V. M. Role of glycolysis in fatty acid and cholesterol synthesis in normal and diabetic rats. Science 1957, **126**, 1012.
2. Stetten, D., Jr., and Boxer, G. E. Studies in carbohydrate metabolism. III. Metabolic defects in alloxan diabetes. J. biol. Chem. 1944, **156**, 271.
3. Chernick, S. S., Chaikoff, I. L., Masoro, E. J., and Isaeff, Eugenia. Lipogenesis and glucose oxidation in the liver of the alloxan-diabetic rat. J. biol. Chem. 1950, **186**, 527.
4. Brady, R. O., and Gurin, S. Biosynthesis of labeled fatty acids and cholesterol in experimental diabetes. J. biol. Chem. 1950, **187**, 589.
5. Van Bruggen, J. T., Yamada, P., Hutchens, T. T., and West, E. S. Lipogenesis of the intact alloxan-diabetic rat. J. biol. Chem. 1954, **209**, 635.
6. Masoro, E. J., Chaikoff, I. L., Chernick, S. S., and Felts, J. M. Previous nutritional state and glucose conversion to fatty acids in liver slices. J. biol. Chem. 1950, **185**, 845.
7. Lyon, I., Masri, M. S., and Chaikoff, I. L. Fasting and hepatic lipogenesis from C^{14}-acetate. J. biol. Chem. 1952, **196**, 25.
8. Wyshak, G. H., and Chaikoff, I. L. Metabolic defects in the liver of fasted rats as shown by utilization of C^{14}-labeled glucose and fructose. J. biol. Chem. 1953, **200**, 851.
9. Chernick, S. S., and Chaikoff, I. L. Insulin and hepatic utilization of glucose for lipogenesis. J. biol. Chem. 1950, **186**, 535.
10. Tepperman, J., Tepperman, H. M., Wilson, M., and DeWitt, J. M. Effect of food intake on lipogenesis by liver slices in vitro. Fed. Proc. 1957, **16**, 128.
11. Tomkins, G. M., and Chaikoff, I. L. Cholesterol synthesis by liver. I. Influence of fasting and of diet. J. biol. Chem. 1952, **196**, 569.
12. Hotta, S., and Chaikoff, I. L. Cholesterol synthesis from acetate in the diabetic liver. J. biol. Chem. 1952, **198**, 895.
13. Muntz, J. A., and Murphy, J. R. The metabolism of variously labeled glucose in rat liver in vivo. J. biol. Chem. 1957, **224**, 971.
14. Wenner, C. E., and Weinhouse, S. An isotope tracer study of glucose catabolism pathways in liver. J. biol. Chem. 1956, **219**, 691.
15. Bloom, B., and Stetten, D., Jr. The fraction of glucose catabolized via the glycolytic pathway. J. biol. Chem. 1955, **212**, 555.
16. Katz, J., Abraham, S., Hill, R., and Chaikoff, I. L. The occurrence and mechanism of the hexose monophosphate shunt in rat liver slices. J. biol. Chem. 1955, **214**, 853.
17. Agranoff, B. W., Brady, R. O., and Colodzin, M. Differential conversion of specifically labeled glucose to $C^{14}O_2$. J. biol. Chem. 1954, **211**, 773.
18. Ashmore, J., Kinoshita, J. H., Nesbett, F. B., and Hastings, A. B. Studies on carbohydrate metabolism in rat liver slices. VIII. Evaluation of the Embden-Meyerhof and phosphogluconate oxidation pathways. J. biol. Chem. 1956, **220**, 619.
19. Siperstein, M. D., and Fagan, V. M. Studies on the relationship between glucose oxidation and intermediary metabolism. II. The role of glucose oxidation in lipogenesis in diabetic rat liver. J. clin. Invest. 1958, **37**, 1196.
20. Wenner, C. E., Dunn, D. F., and Weinhouse, S. A study of glucose oxidation in whole tissue homogenates. J. biol. Chem. 1953, **205**, 409.

21. Siperstein, M. D., and Fagan, V. M. The influence of pyridine nucleotides on the pathways of glucose oxidation. Submitted for publication.
22. Dounce, A. L., Witter, R. F., Monty, K. J., Pate, S., and Cottone, M. A. A method for isolating intact mitochondria and nuclei from the same homogenate, and the influence of mitochondrial destruction on the properties of cell nuclei. J. biophys. biochem. Cytol. 1955, **1**, 139.
23. Baruch, H., and Chaikoff, I. L. A simplified method for determination of lipide-C^{14} in liver. Proc. Soc. exp. Biol. (N. Y.) 1954, **86**, 97.
24. Passmann, J. M., Radin, N. S., and Cooper, J. A. D. Liquid scintillation technique for measuring carbon-14-dioxide activity. Analyt. Chem. 1956, **28**, 484.
25. Sperry, W. M., and Webb, M. A revision of the Schoenheimer-Sperry method for cholesterol determination. J. biol. Chem. 1950, **187**, 97.
26. Langdon, R. G. The requirement of triphosphopyridine nucleotide in fatty acid synthesis. J. Amer. chem. Soc. 1955, **77**, 5190.
27. Siperstein, M. D. Role of glycolysis in cholesterol synthesis. Amer. J. Med. 1957, **22**, 974.
28. Baker, N., Shreeve, W. W., Shipley, R. A., Incefy, G. E., and Miller, M. C^{14} studies in carbohydrate metabolism. I. The oxidation of glucose in normal human subjects. J. biol. Chem. 1954, **211**, 575.
29. Andres, R., Cader, G., and Zierler, K. L. The quantitatively minor role of carbohydrate in oxidative metabolism by skeletal muscle in intact man in the basal state. Measurements of oxygen and glucose uptake and carbon dioxide and lactate production in the forearm. J. clin. Invest. 1956, **35**, 671.
30. Van Baalen, J., and Gurin, S. Cofactor requirements for lipogenesis. J. biol. Chem. 1953, **205**, 303.
31. Tchen, T. T., and Bloch, K. On the conversion of squalene to lanosterol in vitro. J. biol. Chem. 1957, **226**, 921.
32. Seubert, W., Greull, G., and Lynen, F. Die synthese der fettsäuren mit gereinigten enzymen des fettsäurecyclus. Angewandte Chem. 1957, **69**, 359.
33. Brady, R. O., Mamoon, A. M., and Stadtman, E. R. The effects of citrate and coenzyme A on fatty acid metabolism. J. biol. Chem. 1956, **222**, 795.
34. Langdon, R. G. The biosynthesis of fatty acids in rat liver. J. biol. Chem. 1957, **226**, 615.
35. Dituri, F., Shaw, W. N., Warms, J. V. B., and Gurin, S. Lipogenesis in particle-free extracts of rat liver. I. Substrates and cofactor requirements. J. biol. Chem. 1957, **226**, 407.
36. Hele, P., Popjak, G., and Lauryssens, M. Biosynthesis of fatty acids in cell-free preparations. 4. Synthesis of fatty acids from acetate by a partially purified enzyme system from rabbit mammary gland. Biochem. J. 1957, **65**, 348.
37. Lynen, F. Functional group of coenzyme A and its metabolic relations, especially in the fatty acid cycle. Fed. Proc. 1953, **12**, 683.
38. Green, D. E. Oxidation and synthesis of fatty acids in soluble enzyme systems of animal tissues. Clin. Chem. 1955, **1**, 53.
39. Rabinowitz, J. L., and Gurin, S. Biosynthesis of cholesterol and β-hydroxy-β-methylglutaric acid by extracts of liver. J. biol. Chem. 1954, **208**, 307.
40. Dituri, F., Cobey, F. A., Warms, J. V. B., and Gurin, S. Terpenoid intermediates in the biosynthesis of cholesterol. J. biol. Chem. 1956, **221**, 181.
41. Bucher, N. L. R. The formation of radioactive cholesterol and fatty acids from C^{14}-labeled acetate by rat liver homogenates. J. Amer. chem. Soc. 1953, **75**, 498.
42. Hoberman, H. D. Utilization of hydrogen of substrates of pyridine-nucleotide-linked dehydrogenases in reductive biosyntheses. Fed. Proc. 1957, **16**, 197.

STUDIES ON THE RELATIONSHIP BETWEEN GLUCOSE OXIDATION AND INTERMEDIARY METABOLISM. II. THE ROLE OF GLUCOSE OXIDATION IN LIPOGENESIS IN DIABETIC RAT LIVER[1,2]

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In the preceding paper a study was made of the relative importance of the two known glycolytic pathways in controlling lipid synthesis in the liver of normal rats (2). It was concluded that stimulation of fatty acid synthesis observed during accelerated glucose breakdown in normal animals is due primarily to that fraction of the glucose which uses the hexosemonophosphate (HMP) shunt. The Embden-Meyerhof (EM) route of glycolysis, though more important from the standpoint of glucose catabolism, appears to play a relatively minor role in the control of lipogenesis.

The purpose of the investigation to be reported here was to determine whether the depression in fatty acid synthesis which is known to accompany the diabetic state is due to a deficiency of HMP glycolysis or of EM glycolysis.

METHODS

The treatment of the animals, preparation of homogenates and analytical methods were similar to those used in the preceding paper (2). Diabetes was induced by the intravenous injection of 2 per cent alloxan monohydrate in a dosage of 45 mg. per kilogram buffered as described by Klebanoff and Greenbaum (3). The diabetic rats used in these studies had fasting blood sugars of over 250 mg. per cent and excreted urine volumes of over 200 ml. per day while on the high glucose diet.

RESULTS

Lipogenesis in homogenates of diabetic liver

Impaired fatty acid synthesis in diabetic animals, a defect which has been previously shown in both the intact animal using D_2O (4) and in diabetic liver slices with labeled acetate (5, 6), is also demonstrable in liver homogenates (Table I). Unsupplemented homogenates of normal rat livers have consistently been found to synthesize fatty acids at significant rates (2); however, in liver homogenates from diabetic animals lipogenesis is usually markedly depressed, often to the point that it cannot be detected. The synthetic ability of diabetic homogenates averaged 0.53 mμM of added acetate-1-C^{14} incorporated into fatty acids per hour; the corresponding figure for normal livers was 5.1 mμM (2). A similar observation has been previously made by Shaw and Gurin (7).

Effect of repairing glycolytic pathways on diabetic lipogenesis

Glucose oxidation over both the Embden-Meyerhof and the hexosemonophosphate pathways is known to be depressed in diabetes; however, the relationship between these glycolytic defects and the blockage of lipogenesis in diabetes has previously been obscure.

To determine which of these two glycolytic routes is responsible for the lipogenic block in diabetes, glucose oxidation was alternately restored over each of the two glycolytic pathways, as previously described (8), and the effect on lipogenesis was simultaneously measured by observing the incorporation of acetate-1-C^{14} into fatty acids.

Reinstitution of EM glycolysis in diabetic liver caused a relatively slight increase in fatty acid synthesis of zero to twenty-seven fold, averaging 11.2 (Table I). In contrast, however, a very marked repair of lipogenesis was observed when glucose oxidation via the HMP pathway was restored. This synthesis amounted to from 3 to 730 (average, 91) times that produced by reinstitution of the EM pathway and represents a restoration of diabetic lipogenesis to a level well above that seen in normal control homogenates.

[1] A brief report of these experiments has been previously published (1).

[2] These studies were supported by grants from the American Heart Association, the Dallas Heart Association, and the National Heart Institute, United States Public Health Service.

TABLE I

Influence of glycolytic pathways upon lipid synthesis in diabetic livers *

Experiment number	Glycolytic pathway stimulated	Acetate-1-C^{14} converted to: Fatty acid	Cholesterol	CO_2	Experiment number	Glycolytic pathway stimulated	Acetate-1-C^{14} converted to: Fatty acid	Cholesterol	CO_2
		mμM	mμM ×10⁻²	mμM			mμM	mμM ×10⁻²	mμM
1	Neither	<0.1	<3	3	7	Neither	0.6	<3	25
	HMP	73	302	84		HMP	130	3,052	168
	EM	<0.1	<3	4		EM	15	<3	95
	HMP+EM	302	309	68		HMP+EM	159	2,577	138
2	Neither	<0.3	<6	4	8	Neither	0.3	5	9
	HMP	28	62	111		HMP	34	494	76
	EM	1.2	<6	6		EM	8		63
	HMP+EM	263	52	85		HMP+EM	26	205	67
3	Neither	<0.3	<6	3	9	Neither	0.3	<3	8
	HMP	30	754	76		HMP	45	237	49
	EM	0.6	<6	3		EM	4	<3	36
	HMP+EM	88	1,066	96		HMP+EM	58	166	54
4	Neither	<0.3	<5	5	10	Neither	0.7	<3	10
	HMP	38	<5	91		HMP	85	49	38
	EM	<0.3	<5	2		EM	14	<3	27
	HMP+EM	78	<5	67		HMP+EM	99	16	36
5	Neither	0.8	10	6	11	Neither	0.4	<3	9
	HMP	35	2,100	75		HMP	126	234	64
	EM	8	10	63		EM	5	<3	40
	HMP+EM	74	936	82		HMP+EM	55	26	28
6	Neither	0.3	<5	6	12	Neither	2	<3	43
	HMP	12	286	51		HMP	37	10	47
	EM	4	<5	53		EM	12	<3	73
	HMP+EM	21	52	45		HMP+EM	40	3	52

* Cofactor and substrate concentration: glucose-6-phosphate, 18×10^{-3} M; TPN, 0.7×10^{-3} M; DPN, 0.7×10^{-3} M; potassium acetate, 2×10^{-3} M. Total volume in Experiments 1 through 3 was 2.5 ml.; in Experiments 4 through 12 the volume was 1.4 ml.

In fact, reinstitution of HMP glycolysis stimulated the rates of fatty acid synthesis in the diabetic homogenates to the extent (*i.e.*, an average of 189 times the control levels) that, though these values averaged less, they fell within the range of those for *HMP-stimulated* lipogenesis in normal homogenates (2). As in the case of normal liver the simultaneous stimulation of both pathways of glycolysis usually caused a maximal increase in lipogenesis.

The deficient cofactor in diabetic lipogenesis

Since reduced triphosphopyridine nucleotide (TPNH) was found to mediate the stimulatory effects of HMP glycolysis on lipid synthesis in normal liver, it seemed possible that a *deficiency* of this cofactor might be responsible for the lipogenic lesion of diabetes. An attempt was therefore made to repair this lesion by means of an alternate TPNH generating system, isocitrate and triphosphopyridine nucleotide (TPN). The data in Table II demonstrate that this source of TPNH will correct the defect in diabetic lipogenesis at least as well as will the HMP shunt. The addition to the system of either TPN or isocitrate alone, while increasing fatty acid synthesis, had much less effect than did the complete generating system.

Cholesterol synthesis

Cholesterol synthesis in diabetic liver was found to be considerably stimulated by HMP glycolysis (Table I), and in the case of lipogenesis, the alternate TPNH generating system was successfully substituted for HMP oxidation (Table II). TPN without additional substrate, however, caused a very marked stimulation of cholesterol synthesis and it is likely, as was discussed in the previous report (2), that this reflects the require-

TABLE II

Influence of reduced triphosphopyridine nucleotide (TPNH) generating systems upon fatty acid and cholesterol synthesis in diabetic liver

Experiment	Cofactor and/or substrate	Acetate-C^{14} converted to: Fatty acids	Cholesterol
		mμM	*mμM* $\times 10^{-2}$
1	Glucose-6-phosphate	0.5	<5
	Potassium isocitrate	7.3	<5
	TPN+glucose-6-phosphate	40.6	99
	TPN+potassium isocitrate	71.2	36
	TPN	10.9	73
2	Glucose-6-phosphate	0.1	<2
	Potassium isocitrate	7.2	<2
	TPN+glucose-6-phosphate	26.1	330
	TPN+potassium isocitrate	99.5	140
	TPN	0.8	340

Concentration of additions:
Experiment 1. TPN, 0.8×10^{-3} M; glucose-6-phosphate, 21×10^{-3} M; potassium isocitrate, 21×10^{-3} M in a total volume of 2.7 ml.
Experiment 2. TPN, 0.7×10^{-3} M; glucose-6-phosphate, 18×10^{-3} M; potassium isocitrate, 2×10^{-3} M in a total volume of 1.4 ml.

ment for a lesser concentration of TPNH for cholesterolgenesis than for lipogenesis.

$C^{14}O_2$ production

In all but one case (Experiment 12, Table I), supplementation with TPN caused a marked increase in the production of $C^{14}O_2$ from acetate-1-C^{14}. Diphosphopyridine nucleotide (DPN) usually caused a significant stimulation, though of somewhat smaller magnitude than was seen with TPN. The response to the addition of both cofactors was not significantly greater than that usually produced by either TPN or by DPN alone.

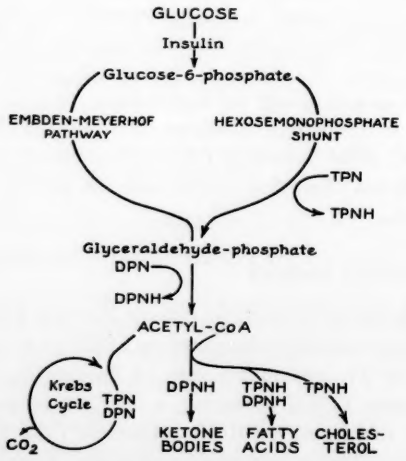

FIG. 1. A SUMMARY OF GLYCOLYTIC PATHWAYS AND THEIR ARTICULATION, ESPECIALLY VIA PYRIDINE NUCLEOTIDES, WITH SEVERAL OTHER INTRACELLULAR FUNCTIONS

DISCUSSION

The nature of the lipogenic defect in diabetes has been thoroughly discussed in recent reviews by Chaikoff (9) and by Stadie (10). It has been clear for some years that this lesion is in some manner due to a lack of insulin action since, as was shown by Chernick and Chaikoff (11), the administration of this hormone to diabetic animals will promptly restore lipogenesis to normal or above-normal levels. These authors have concluded that the inability of the diabetic animal to convert glucose into fatty acids cannot be explained solely on the basis of a single metabolic block, presumably involving the phosphorylation of glucose, and they have suggested the existence of a second block, located on the pathway of synthesis of fatty acids from a two-carbon compound (acetylcoenzyme A). Figure 1 summarizes this and the other relationships between glycolysis and the metabolic processes, to be discussed below.

Baker, Chaikoff, and Schusdek (12) subsequently demonstrated that fatty acid synthesis in the diabetic could be restored to normal simply by prefeeding with fructose, a sugar which does not require insulin for its metabolism. The diabetic defect in lipogenesis is therefore not due to a lack of a direct insulin stimulation of lipogenesis but is in fact secondary to the decrease in glycolysis. This conclusion was further supported by the finding of Shaw and Gurin that lipogenesis in diabetic homogenates could be in part corrected by the addition of a number of those glycolytic intermediates involved beyond the insulin sensitive step in glucose metabolism (7). It seemed likely, therefore, that some factor normally produced during the process of glycolysis serves to maintain normal fatty acid synthesis and that this factor is then lacking in the diabetic state.

The first object of the present study was to determine whether a deficiency of one of the two pathways of glycolysis, *i.e.*, the Embden-Meyerhof

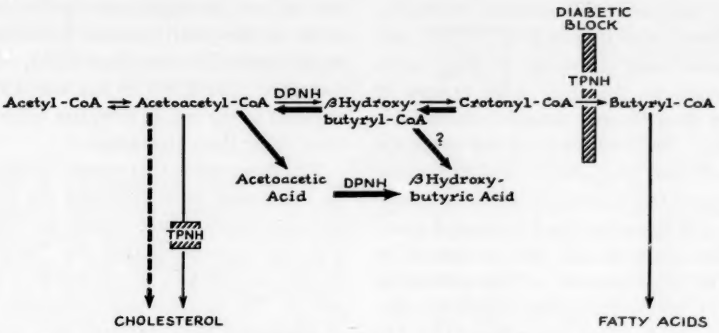

FIG. 2. AN EXPLANATION, BASED ON A TPNH DEFICIENCY, OF SOME OF THE METABOLIC LESIONS OF DIABETES

route or the hexosemonophosphate shunt, is responsible for the lipogenic lesion in diabetes.

Despite the fact that the EM pathway is normally the quantitatively more important pathway, the results given in Table I indicate that reinstitution of EM glycolysis does not significantly stimulate lipogenesis in diabetic livers. On the other hand, restoration of HMP glycolysis caused a very significant increase in lipogenesis, the HMP route being approximately ninetyfold more effective than the EM pathway.

It is concluded from these experiments, therefore, that the block in lipogenesis of diabetic liver can be repaired simply by reinstitution of HMP glycolysis and it follows that this lesion in fatty acid synthesis may be ascribed in the diabetic primarily to a lack of glucose oxidation via the hexosemonophosphate pathway. The cofactors generated by EM glycolysis, though doubtless necessary for lipogenesis, would appear from our data to be supplied in adequate amounts despite the marked reduction in this glycolytic system in the diabetic state, or else, as will be discussed below, such factors are amply provided to the cell by reactions other than glycolysis. On the other hand, since it is clear from the results of this study that a deficiency in the HMP shunt does markedly inhibit lipogenesis, the lack of a factor normally supplied by this reaction is presumably chiefly responsible for the defective lipogenesis of diabetes.

The nature of this cofactor was suggested by the demonstration that HMP glycolysis is not unique in its ability to repair the lipogenic lesion of diabetes. Isocitrate and TPN, another system which, like HMP glycolysis, produces TPNH, is also capable of restoring lipogenesis in diabetic liver. It is likely, therefore, that the inability of the diabetic to synthesize fatty acids is due specifically to a deficiency of the coenzyme, TPNH.

The site of action of TPNH in the process of fatty acid synthesis, as has been shown by Langdon, is probably at the conversion of crotonyl-CoA to butyryl-CoA (13). Our demonstration that TPNH is the cofactor whose deficiency appears to be primarily responsible for the block in diabetic lipogenesis therefore locates the site of the diabetic lesion at this reductive step in the pathway of fatty acid synthesis (Figure 2). The recent finding of Shaw, Dituri, and Gurin (14) that butyryl-CoA will stimulate fatty acid synthesis in diabetic liver indicated that this lesion is located at some point prior to the synthesis of butyryl-CoA and so is consistent with the site of the block suggested here. However, for reasons that are not apparent these investigators were unable to show an effect of TPNH on lipogenesis.

The location of the major lipogenic defect in diabetes at the point of conversion of crotonyl-CoA to butyryl-CoA is also consistent with a number of the clinical observations of diabetic ketosis and in addition offers an explanation for the nature of the organic acids which appear in this derangement. The two four-carbon ketone bodies which accumulate in uncontrolled diabetes are acetoacetic acid and β-hydroxybutyric acid. These are two of the acids whose activated derivatives are required in fatty acid synthesis before the block at the site of TPNH action (Figure 2). We would suggest

that these two activated acids increase in concentration in diabetes because a lack of TPNH prevents them from being converted to fatty acids, and as illustrated by the thick solid arrows in Figure 2, they then become dammed behind this metabolic block. The hydrolysis of the coenzyme A from each of these compounds could then cause an accumulation of free acetoacetic and β-hydroxybutyric acids. On the other hand, increased quantities of β-hydroxybutyric acid can be formed by reduction of the large amounts of *free* acetoacetic acid (Figure 2), and in fact there is evidence that this is the major route of its production (15). In either case, as a result of the TPNH block, the two acids actually observed in excess in diabetic ketosis would be expected to accumulate. Crotonyl-CoA, the compound occurring just prior to the TPNH block, might not increase in concentration in diabetes since the equilibrium between it and β-hydroxybutyryl-CoA is known to favor the latter (16), and this fact may account for the probable absence of pathological quantities of free crotonic acid in diabetic ketosis. The conclusions of our *in vitro* studies can therefore provide an explanation of the nature of diabetic ketosis which is quite consistent with the findings in the clinical state.[3]

In this regard, it should be emphasized that the relative ineffectuality of Embden-Meyerhof glycolysis in correcting the diabetic lesion in our experiments is also consistent with the findings in clinical diabetes ketosis. The diabetic retains the ability to synthesize large quantities of β-hydroxybutyric acid, and this is often the predominant ketone body present in diabetic ketosis. Since formation of β-hydroxybutyric acid requires the action of reduced diphosphopyridine nucleotide (DPNH) (Figure 2), it is unlikely that a lack of this coenzyme, and hence a depression of the Embden-Meyerhof pathway, are responsible for the metabolic lesions of the *intact* diabetic animal. It should be noted that during the *oxidation* of fatty acids one molecule of DPNH is produced for each two carbons removed from the fatty acid molecule, while on the other hand no TPNH is believed to result from these reactions (19). It is possible, therefore, that DPNH but not TPNH may be supplied to the cell in adequate quantities by reactions other than glycolysis.

The observation that repair of diabetic lipogenesis will result from restoring the function of the hexosemonophosphate shunt is fully consistent with the conclusion that the "second" block in diabetic metabolism is not due to a lack of insulin *per se*. The location and mechanism of this lesion, as suggested here, indicate that both this defect in lipogenesis and the resulting ketosis of diabetes are *secondary* to the depressed glucose oxidation. It follows therefore that these lesions can probably all be explained on the basis of a single action of insulin at the site of the initial phosphorylation of glucose.

Whether a deficiency of insulin causes a greater depression of HMP glycolysis than of EM glycolysis, as has been suggested by recent studies (20–22), remains to be finally decided (23). A selective depression of the HMP pathway in diabetes due to a decrease either of TPN or of HMP enzymes (22, 24) is, however, consistent with the data and concept presented here.

The synthesis of cholesterol was also very greatly increased by reinstitution of HMP glycolysis in the diabetic liver homogenate and a block in this pathway is therefore shown in the scheme of diabetic metabolism, illustrated in Figure 2. The abnormally *elevated* rates of cholesterol synthesis which have been noted in diabetic liver slices (25) were not observed in the present studies. Our data suggest, however, that the synthesis of cholesterol may require smaller concentrations of TPNH than does fatty acid synthesis (2) (Table II). It is possible, therefore, that cholesterol synthesis is less affected by a deficiency of TPNH. The increased cholesterolgenesis seen in diabetic liver might then be due to the stimulatory effect of acetoacetyl-CoA accumulation; this possibility is indicated by the thick dotted arrow in Figure 2.

Finally, in contrast to the case in normal liver (2), a stimulation of the Krebs cycle was regularly obtained by the addition of pyridine nucleotides to diabetic liver. This observation is consistent with the lower levels of pyridine nucleotides found in diabetic liver by Glock and McLean (24) and sug-

[3] A liver enzyme system has recently been purified (17) which is capable of forming fatty acids using TPNH as the sole reducing coenzyme (18). In this system a deficiency of TPNH might then be manifest as a block in the conversion of acetoacetyl-CoA to β-hydroxybutyryl-CoA. Acetoacetic and β-hydroxybutyric acid would then accumulate as described above.

gests that availability of pyridine nucleotides may become a controlling factor in the operation of the Krebs cycle in the diabetic cell. Further studies of this phenomenon are in progress.

SUMMARY

1. The cause of the lipogenic defect which accompanies diabetes has been investigated in cell-free liver preparations from alloxan diabetic rats.

2. It is concluded that this metabolic lesion is due primarily to a deficiency of glucose oxidation via the hexosemonophosphate pathway of glycolysis; the lack of glycolysis via the Embden-Meyerhof route, which also accompanies the diabetic state, apparently plays a minor role in depressing lipogenesis.

3. Evidence is presented which indicates that a deficiency of the reduced triphosphopyridine nucleotide (TPNH), normally produced by the hexosemonophosphate route, is the specific cause of the lipogenic defect in diabetes.

4. A concept of the nature of diabetic ketosis is presented based on a block at the site of action of TPNH in fatty acid synthesis.

REFERENCES

1. Siperstein, M. D., and Fagan, V. M. Role of glycolysis in fatty acid and cholesterol synthesis in normal and diabetic rats. Science 1957, **126**, 1012.
2. Siperstein, M. D., and Fagan, V. M. Studies on the relationship between glucose oxidation and intermediary metabolism. I. The influence of glycolysis on the synthesis of cholesterol and fatty acids in normal liver. J. clin. Invest. 1958, **37**, 1185.
3. Klebanoff, S. J., and Greenbaum, A. L. The effect of pH on the diabetogenic action of alloxan. J. Endocr. 1954, **11**, 314.
4. Stetten, D., Jr., and Boxer, G. E. Studies in carbohydrate metabolism. III. Metabolic defects in alloxan diabetes. J. biol. Chem. 1944, **156**, 271.
5. Brady, R. O., and Gurin, S. Biosynthesis of labeled fatty acids and cholesterol in experimental diabetes. J. biol. Chem. 1950, **187**, 589.
6. Chernick, S. S., and Chaikoff, I. L. Two blocks in carbohydrate utilization in the liver of the diabetic rat. J. biol. Chem. 1951, **188**, 389.
7. Shaw, W., and Gurin, S. Relationship of glycolysis to lipogenesis in aqueous extracts of liver. Arch. Biochem. 1953, **47**, 220.
8. Siperstein, M. D., and Fagan, V. M. The influence of pyridine nucleotides on the pathways of glucose oxidation. Submitted for publication.
9. Chaikoff, I. L. Metabolic blocks in carbohydrate metabolism in diabetes. The Harvey Lectures, 1951–1952. New York, Academic Press Inc., 1953, vol. 47, p. 99.
10. Stadie, W. C. Current concepts of the action of insulin. Physiol. Rev. 1954, **34**, 52.
11. Chernick, S. S., and Chaikoff, I. L. Insulin and hepatic utilization of glucose for lipogenesis. J. biol. Chem. 1950, **186**, 535.
12. Baker, N., Chaikoff, I. L., and Schusdek, A. Effect of fructose on lipogenesis from lactate and acetate in diabetic liver. J. biol. Chem. 1952, **194**, 435.
13. Langdon, R. G. The requirement of triphosphopyridine nucleotide in fatty acid synthesis. J. Amer. chem. Soc. 1955, **77**, 5190.
14. Shaw, W. N., Dituri, F., and Gurin, S. Lipogenesis in particle-free extracts of rat liver. II. Experimental diabetes. J. biol. Chem. 1957, **226**, 417.
15. Lehninger, A. L., and Greville, G. D. The enzymic oxidation of d- and l-β-hydroxybutyrate. Biochim. Biophys. Acta 1953, **12**, 188.
16. Stern, J. R., and del Campillo, A. Enzymes of fatty acid metabolism. II. Properties of crystalline crotonase. J. biol. Chem. 1956, **218**, 985.
17. Wakil, S. J., Porter, J. W., and Gibson, D. M. Studies on the mechanism of fatty acid synthesis. I. Preparation and purification of an enzyme system for reconstruction of fatty acid synthesis. Biochim. Biophys. Acta 1957, **24**, 453.
18. Titchener, E. B., Gibson, D. M., and Wakil, S. J. Requirements for fatty acid biosynthesis. Fed. Proc. 1958, **17**, 322.
19. Green, D. E. Oxidation and synthesis of fatty acids in soluble enzyme systems of animal tissues. Clin. Chem. 1955, **1**, 53.
20. Bloom, B. Fraction of glucose catabolized via the Embden-Meyerhof pathway: Alloxan-diabetic and fasted rats. J. biol. Chem. 1955, **215**, 467.
21. Felts, J. M., Doell, R. G., and Chaikoff, I. L. The effect of insulin on the pathways of conversion of glucose to fatty acids in the liver. J. biol. Chem. 1956, **219**, 473.
22. Glock, G. E., and McLean, P. A preliminary investigation of the hormonal control of the hexose monophosphate oxidative pathway. Biochem. J. 1955, **61**, 390.
23. Ashmore, J., Cahill, G. F., Jr., Hastings, A. B., and Zottu, S. Studies on carbohydrate metabolism in rat liver slices. VIII. Effect of ions and hormones on pathways of glucose-6-phosphate metabolism. J. biol. Chem. 1957, **224**, 225.
24. Glock, G. E., and McLean, P. Effects of hormones on levels of oxidized and reduced diphosphopyridine nucleotide and triphosphopyridine nucleotide in liver and diaphragm. Biochem. J. 1955, **61**, 397.
25. Hotta, S., and Chaikoff, I. L. Cholesterol synthesis from acetate in the diabetic liver. J. biol. Chem. 1952, **198**, 895.

THE DESTRUCTION OF RED CELLS BY ANTIBODIES IN MAN. II. PYROGENIC, LEUKOCYTIC AND DERMAL RESPONSES TO IMMUNE HEMOLYSIS[1]

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The immune destruction of red cells is usually mediated by the filtration of agglutinated, or agglutinable, red cells (1, 2). Initially red cell agglutination is enhanced in the circulation by the action of certain hydrophilic macromolecules, most notably fibrinogen, which induce rouleaux formation and red cell sedimentation (2, 3). Strongly, or coarsely, agglutinated red cells are largely trapped in the liver and lungs, whereas weakly, or finely, agglutinated cells are trapped mainly by the spleen (2). The greater proficiency of the spleen in filtering agglutinable, antibody-coated, red cells, as well as red cells which have been altered slightly in size or shape, may be based entirely upon the physical relation between the size of the cell particle and the pore size and perfusion pressure of essentially mechanical filters (4). The living filter beds of the reticuloendothelial system are presumably scoured by the phagocytic activity of macrophages, and indeed the lysis of red cells coated with nonhemolyzing (anti-D) antibodies appears to occur within a few minutes of their sequestration in the spleen (2).

In the course of making certain of the observations summarized above, which involved the injection of small volumes of incompatible red cells into normal subjects, an interesting pattern of host response to the injected cells emerged. It is well known that acute blood destruction may provoke an assortment of clinical reactions, including pain (particularly in the precordium and low back), dyspnea, coughing, flushing, chills, fever, nausea and vomiting, as well as certain allergic manifestations such as urticaria and even serum sickness (5). The pathogenesis of these phenomena, including the chills and fever of acute hemolytic episodes and the milder protracted fever which may attend chronic hemolytic states, is obscure. Marked alterations in blood leukocyte levels are also frequent in hemolytic disorders. Relatively little exact information exists concerning the acute phenomena partly because most observations have been made during transfusion reactions in which initial changes were not observed and in which the effects of contaminating bacterial pyrogens, shock, sensitivity to the buffy coat (6), and so forth could not be excluded. Nevertheless, an abrupt leukopenia was early found to occur during paroxysmal cold hemoglobinuria (7, 8), and leukopenia was observed following the injection of isoantibodies into dogs (9) and of incompatible blood into human subjects (10). More recently Swisher (11) described leukopenia and thrombocytopenia in dogs given incompatible blood in those instances where complement was fixed; and mixed agglutinates of red cells, leukocytes and platelets were noted, both *in vivo* (11) and *in vitro* (12). In the present studies, preliminarily reported elsewhere (13), fever and leukopenia were found to be characteristic of the normal host response to immune hemolysis and analogous in their pathogenesis to the host responses to other antigen-antibody reactions and to bacterial endotoxins.

METHODS

Techniques of preparing red cells and antibodies for injection. In most experiments whole blood was drawn into sterile syringes and transferred to screw-capped glass tubes containing one-fourth volume of acid-citrate-dextrose (ACD) solution. Following experimental manipulations *in vitro* the cells were washed twice in 10 to 20 volumes of physiologic saline before resuspension in saline and injection. The saline and anticoagulant solutions employed were sterile and demonstrated (in rabbits and/or in human subjects) to be pyrogen-free. Plasma contain-

[1] This investigation was supported in part by a grant from the Helen Hay Whitney Foundation and by Research Grant No. RG3507 (C_8) from the National Institutes of Health, Public Health Service.

ing red cell antibodies was obtained by drawing whole blood into sterile plastic equipment containing either ACD or ethylenediaminetetracetic acid (EDTA) as anticoagulant; the blood was centrifuged and the plasma transferred to a second plastic bag via a closed system for storage at − 20° C. until used.[2] Again, the sterility and nonpyrogenicity of equipment and anticoagulant solutions were verified before use. Red cell sensitization with anti-D *in vitro* was achieved by incubating each volume of red cells in two volumes of physiologic saline with two volumes of anti-D plasma for one hour at 37° C., after which the cells were washed as described above.

Techniques involving Cr^{51}. In most experiments red cells were labeled with $Na_2Cr^{51}O_4$,[3] as described previously (2), before being washed as described above. The suspensions were injected intravenously into human subjects within a period of one minute. Thereafter specimens of blood were drawn periodically from each subject into saline-wet syringes and then were divided into two parts. One part, which was mixed with dry "balanced" oxalate, was later lysed by freezing and thawing to provide samples for measuring whole blood radioactivity. The other part, mixed with one-tenth of its volume of 3 per cent sodium citrate solution, was centrifuged, and the supernatant plasma recentrifuged, to provide plasma for determination of its radioactivity. The radioactivity of 3 ml. samples of whole blood, plasma and the Cr^{51} standards was determined in a well-type scintillation counter. The distribution of radioactivity at body surface over the subjects' hearts, livers and spleens was determined with a directional scintillation counter as described elsewhere (14).

Leukocyte measurements. Leukocytes were counted by standard techniques (15) in samples pipetted from oxalated blood specimens drawn within a few seconds of venesection. The pipetting was performed promptly for theoretical reasons, although enumeration of leukocytes pipetted from the same oxalate specimens two to three hours later in several experiments revealed that their leukocyte counts remained constant within that period of time *ex vivo*. Differential leukocyte counts were made on cover slip smears of blood obtained from the needle tip and stained with Wright's stain. Unless a scarcity of cells prevented it, 2,000 leukocytes were examined during each differential count.[4] Direct eosinophil counts were made, using Randolph's solution (15).

Miscellaneous techniques. Body temperatures were determined rectally at frequent intervals during base line (control) measurements, with the subject resting in bed for 30 minutes or more, and during the experimental period. Plasma hemoglobin levels were determined by a photo-electric adaptation of the benzidine technique (15), using the same specimens employed for measuring plasma Cr^{51}. The Coombs (antiglobulin) test (16) was performed by the tube technique and the polyvinylpyrrolidone ("P. V. P.") test was carried out as described elsewhere (3). Peripheral blood was searched for the presence of "autoagglutination" by suspending 0.1 ml. of fresh or oxalated blood in 10 ml. of warm saline, and microscopically examining a large drop of this freely-moving cell suspension on a glass slide. The osmotic fragility of red cells (17), the serum bilirubin levels (18), and plasma fibrinogen levels (19) were determined in certain observations.

Human subjects. The normal human subjects who cooperated in this study were either elderly men with minor illnesses or laboratory personnel, in whom no diseases of the hematopoietic or reticuloendothelial systems were believed to be present. Several other subjects were studied who had previously undergone accidental immunization against the D antigen of the Rh system as a result of transfusion or pregnancy.

RESULTS

Effects of ABO-incompatible red cells

The injection of 10 ml. of washed type A or type B red cells into an ABO-incompatible recipient (whether or not he possessed demonstrable isohemolysins) led to the sequestration, largely in the liver, of most of the injected red cells within a few minutes, as determined by measurement of blood and body surface Cr^{51} activity. Simultaneously, as revealed in Figure 1, blood leukocyte levels abruptly fell to about 30 per cent of control levels, remained low for approximately 30 minutes, and then rose to levels somewhat above control levels in two to four hours. Differential leukocyte counts, as in a characteristic study depicted in Figure 2, revealed that leukopenia represented a virtual disappearance of granulocytes and of monocytes from the circulation. In general the levels of mature granulocytes fell more sharply than did those of band forms. The influence of granulocyte maturity upon the granulocytopenic effect of isohemolysis was most clearly evident in a study of a patient with chronic myelogenous leukemia. The injection of ABO-incompatible red cells into this patient caused a pronounced reduction in the level of mature granulocytes, a moderate fall in the level of band forms, but had no effect on the levels of metamyelocytes and myelocytes. In most subjects, levels of mature eosinophils (not shown), measured both directly and indirectly, fell as dramatically as did those of mature neutrophilic

[2] We are grateful to Dr. A. R. Jones and the Blood Grouping Laboratory of Boston, Mass., for their cooperation throughout this study in providing the antisera.

[3] "Rachromate" of high specific activity, Abbott Laboratories, North Chicago, Ill.

[4] We are indebted to Miss Geneva A. Daland, who performed most of these differential counts.

granulocytes. In several experiments basophil levels (not shown) also fell, although the number of cells involved was often too small for certainty. Within 20 minutes of the disappearance of granulocytes and monocytes, neutrophilic band forms (and small numbers of metamyelocytes) began to increase in the circulation, reaching peak levels within two hours of 5 to 20 times preinjection levels, and falling slowly thereafter as an adult neutrophilia predominated. Meanwhile, there was a relatively small rise in eosinophils, and monocyte levels increased and rose very slowly to preinjection levels without an overshoot. Beginning about two hours after the injection of red cells, a moderate decline in lymphocytes was often observed, as in Figure 2, reaching a low point in four hours.

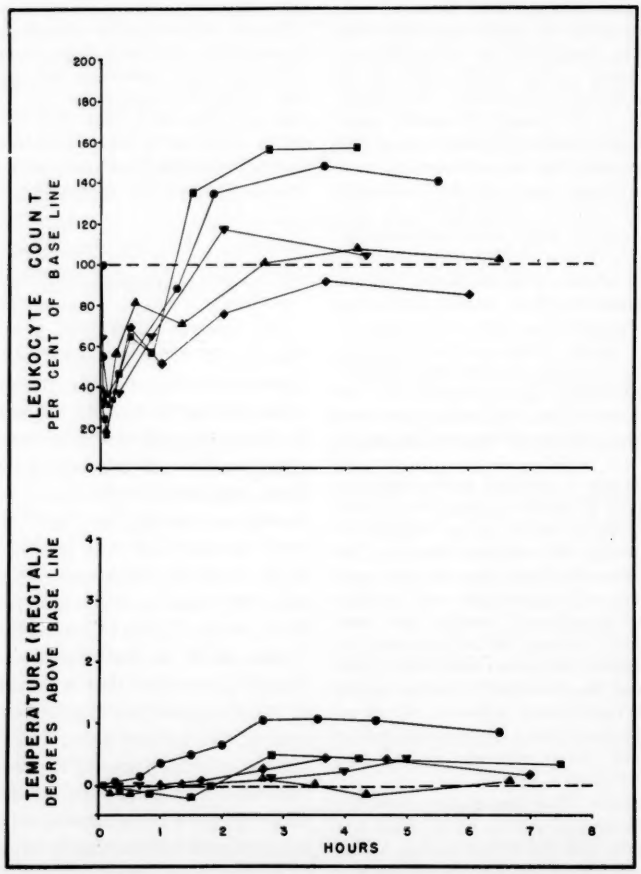

FIG. 1. LEUKOCYTE AND BODY TEMPERATURE CHANGES FOLLOWING INTRAVENOUS INJECTION OF ABO-INCOMPATIBLE RED CELLS

As shown in the upper portion of the figure, blood leukocyte levels of the five normal subjects fell precipitously to about 30 per cent of base line values within two to three minutes. Leukopenia was rather transient and normal, or above normal; leukocyte levels were regained within two or three hours. No chills were observed, and, as shown in the lower portion of the figure, the subjects' rectal temperatures tended to increase only slightly to maximum levels in three or four hours.

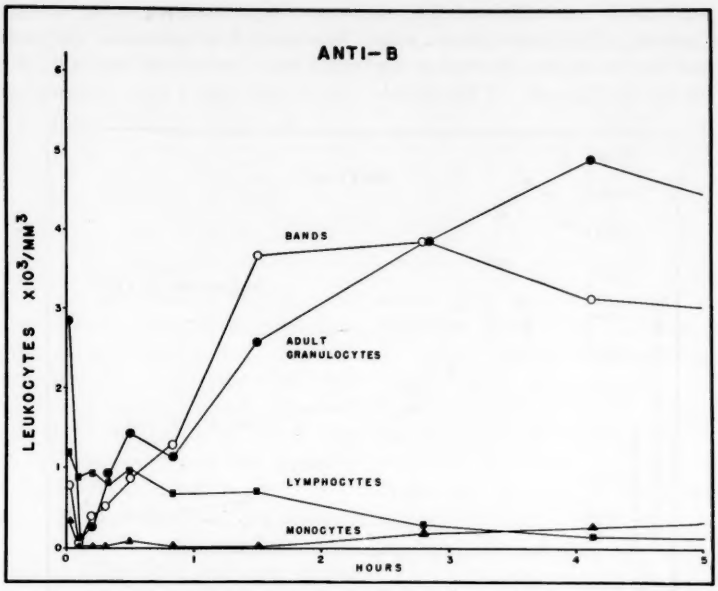

FIG. 2. CHANGES IN LEUKOCYTE DIFFERENTIAL COUNTS FOLLOWING INTRAVENOUS INJECTION OF TYPE B RED CELLS INTO A TYPE O SUBJECT

In this representative study, the abrupt leukopenia portrayed in Figure 1 is seen to result from a virtually complete disappearance of granulocytes and monocytes. Within 30 minutes of the red cell injection, the numbers of band forms began to increase and within two hours reached levels fivefold higher than base line. At the same time adult granulocytes increased to reach mildly elevated levels in about four hours. Note that a monocytosis failed to appear, and that the lymphocyte count was unaffected during the acute hemolysis, but began to decline gradually two hours after the injection.

Usually the leukopenia produced by ABO-incompatible red cells was maximal between three and five minutes after injection. Speed of onset was observed more critically in subjects having an inlying cardiac catheter and a Cournand needle lying in the brachial artery, which thus permitted samplings at approximately 10 second intervals of pulmonary artery blood and brachial artery blood, respectively.[5] One of three such studies is presented in Figure 3. After the catheters had been in place for 30 minutes or more, 10 ml. of Cr^{51}-labeled ABO-incompatible red cells was injected into a leg vein. Leukocyte levels began to fall within a few seconds of the appearance of Cr^{51}-labeled red cells at the sampling sites. There was no definite difference at a given instant between leukocyte levels in the pulmonary artery and in the brachial artery.

The number of ABO-incompatible red cells necessary to produce a definite leukopenia on intravenous injection was determined by injecting several normal subjects with various amounts of red cells which had been freshly drawn in heparinized syringes from incompatible donors and washed twice with saline. As seen in Table I, relatively small numbers of red cells (2.2×10^{10} red cells or less than 2.0 ml. of packed cells) caused a 65 per cent reduction in leukocyte levels and an almost total disappearance of granulocytes. Smaller injections caused lesser degrees of leukopenia, but as few as 5×10^{8} red cells (0.05 ml. of packed cells) caused a significant fall in leukocyte levels. By estimating the number of circulating leuko-

[5] Studies on patients undergoing catheterization of the right side of the heart were possible through the generous cooperation of Dr. Walter Abelmann and his associates.

cytes in these subjects, it was calculated that as many as 15 leukocytes, chiefly granulocytes, may be removed from the recipient's circulation for each incompatible red cell injected. A 10 ml. volume of type O serum, having an agglutinin titer of $\frac{1}{128}$ against a 5 per cent suspension of type A cells and a hemolysin titer of $\frac{1}{4}$, was injected intravenously into a type A normal subject. There

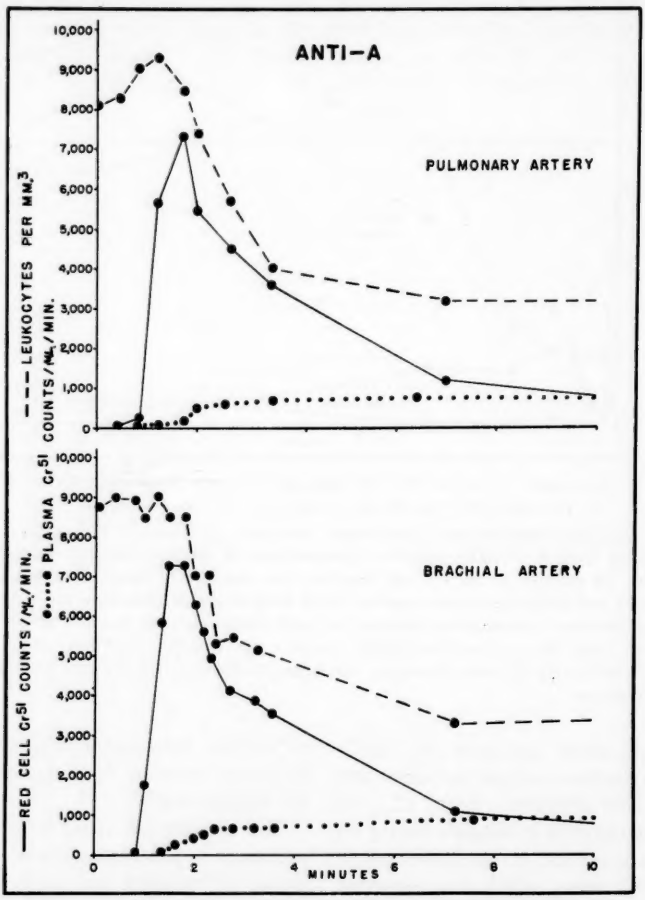

FIG. 3. A COMPARISON OF LEUKOCYTE LEVELS AND RED CELL AND PLASMA RADIOACTIVITY IN PULMONARY ARTERY AND BRACHIAL ARTERY FOLLOWING THE INJECTION OF Cr^{51}-LABELED TYPE A RED CELLS INTO A TYPE O SUBJECT

Within less than a minute of the first appearance of Cr^{51}-labeled red cells at the sampling sites, and within a few seconds of the appearance of Cr^{51} (and of hemoglobin) in the plasma, leukocyte levels (interrupted line) commenced to fall. There was no certain difference in leukocyte levels of blood before and after passage through the lungs. At both sites, leukopenia (interrupted line), Cr^{51}-labeled red cell disappearance (uninterrupted line), and hemoglobinemia (dotted line) became evident almost simultaneously. Note that in this subject, whose serum revealed no isohemolysins on conventional testing *in vitro*, most of the Cr^{51} derived from the incompatible red cells failed to appear in the plasma, indicating that red cell sequestration was the chief mechanism of red cell destruction (2).

TABLE I

The comparative effects on leukocyte levels of intravenous injections of various quantities of ABO-incompatible red cells

Red cell type		Number of red cells injected	Reduction in leukocyte level (*% of base line*)	Time of maximum leukopenia (*min.*)	Estimated ratio white cells removed/red cells injected
Donor	Recipient				
B	A	5.0×10^8	−22	3	14.6
A	O	2.5×10^9	−37	3	6.0
B	A	8.8×10^9	−49	5	2.3
A	O	2.2×10^{10}	−65	5	1.3
B	O	1.2×10^{11}	−70	5	0.2

was no hemolysis or agglutination *in vivo,* presumably because of distribution of the antibodies among an excess of the antigenic red cells, and there was no leukopenia. However, when this same volume of serum was previously mixed *in vitro* with 1 ml. of the subject's type A red cells and the hemolyzed suspension was then injected, an immediate leukopenia followed.

The injection of 10 ml. volumes of Cr^{51}-labeled, washed ABO-compatible red cells into three normal subjects had no effect upon leukocyte levels, nor did leukopenia occur when similar volumes of compatible red cells were injected after they had been hemolyzed by repeated, rapid freezing and thawing. Solutions of hemoglobin derived from water-hemolyzed compatible red cells also had no effect upon leukocyte levels in two normal subjects. In a type O subject, however, the injection of the membranes of 10 ml. of type A red cells, after these cells had been hemolyzed by freezing and thawing and the membranes washed almost free of hemoglobin, caused a marked leukopenia. Several hematologically normal subjects were injected with a preparation of soluble A substance.[6] Six out of seven of the type O subjects developed a definite leukopenia, maximal in three minutes, whereas none of five type A subjects developed a leukopenic response. As with the injection of type A cells, the leukopenia caused by the injection of A substance resulted from a reduction in circulating granulocyte and monocyte levels.

As shown in the lower portion of Figure 1, the injection of ABO-incompatible red cells caused mild increases in the rectal temperatures of the recipients, with an average temperature maximum in five subjects of 0.5° F. above the pre-experimental level between three and four hours after injection. None of these subjects developed chills. In a few subjects transient symptoms appeared within two or three minutes of the injection of incompatible red cells. These symptoms included lumbar pain, dyspnea, hyperperistalsis, emotional instability and, in two instances, marked flushing of the face. In one type O subject transient dyspnea and flushing followed the injection of soluble A substance.

Effects of type D red cells in the presence of anti-D

After the injection of 10 ml. volumes of Cr^{51}-labeled, washed type D red cells into four otherwise normal subjects who possessed incomplete (nonagglutinating) antibodies against type D red cells, these cells were removed from the circulation, but more slowly than was the case with ABO-incompatible red cells. The labeled red cells were largely trapped in the spleen, following which plasma hemoglobin levels increased only slightly and gradually to a maximum about one hour after injection (2). Meanwhile, blood leukocyte levels declined slowly, but profoundly, to their lowest values at the end of about one hour (Figure 4). As with ABO-incompatibility, the leukopenia directly reflected a fall of granulocytes and monocytes, often to the point of their temporary disappearance (Figure 5). Thereafter, band neutrophils (along with younger forms) markedly increased, and later a moderate adult neutrophilia was seen, becoming maximal four to five hours after injection. A striking lymphopenia characteristically began about two hours after injection, reaching lowest values one or two hours thereafter.

A series of observations was then conducted in which normal Cr^{51}-labeled type D red cells were sensitized *in vitro* as was described above and re-

[6] "Blood Group Specific Substance A," derived from porcine gastric mucosa, Knickerbocker Blood Bank, New York, N. Y.

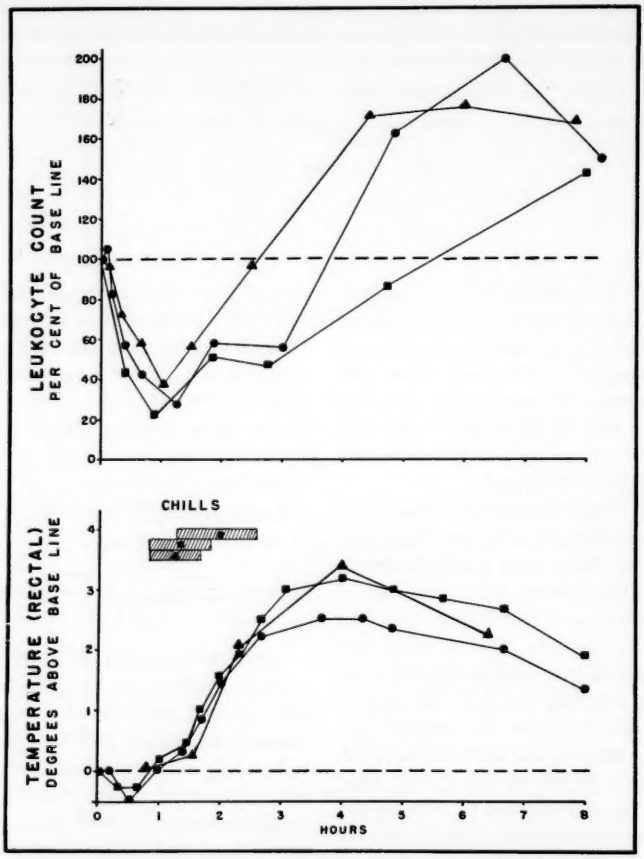

FIG. 4. LEUKOCYTE AND BODY TEMPERATURE CHANGES FOLLOWING INTRAVENOUS INJECTION OF TYPE D RED CELLS INTO D-SENSITIZED SUBJECTS

The upper portion of this figure depicts the resulting slow but marked reduction in the leukocyte levels of each of three subjects whose serum contained incomplete anti-D. Note the characteristic timing of the pyrogenic response. (Similar leukopenic and febrile responses occurred in a fourth subject; however, systematic observations of body temperature levels were not made.)

injected into the donor unless otherwise stated. These washed sensitized red cells were removed from the circulation of the normal D-positive subjects at a rate similar to that of type D red cells injected into sensitized subjects, and in this instance appeared to be sequestered exclusively in the spleen (*2*). Once again, as seen in Figure 6, a slow but marked depression of leukocyte levels occurred, maximal at one hour, following which a moderate neutrophilia developed and reached peak levels four to five hours after injection. As in the previous observations, the leukopenia resulted from a fall in granulocytes and monocytes, and was followed by a band neutrophilia, and two hours after injection by lymphopenia. A virtually identical sequence of events followed the injection of anti-D sensitized red cells into D-negative individuals, whereas the injection of D-negative red

cells labeled with Cr^{51} and incubated with anti-D plasma in the same fashion had no effect upon leukocyte levels.

The temperature responses of D-sensitized subjects receiving type D red cells, and of normal subjects receiving autogenous type D red cells previously sensitized with anti-D *in vitro,* are presented in the lower portion of Figures 4 and 6. No abrupt symptoms, such as occurred in some patients injected with ABO-incompatible red cells, were observed. However, in each situation, and without exception, chills developed between 50 and 75 minutes and lasted for 30 to 60 minutes. The chills were preceded by mild hypothermia, and were often accompanied by nausea, restlessness and occasionally emotional instability. This period was followed by a steady rise in rectal temperatures, to maximum values averaging 3° F. above initial levels between four and five hours after injection.

The intravenous injection of 20 ml. of anti-D plasma into three type D individuals led to smaller rises in plasma hemoglobin and to lesser reductions in leukocyte levels than were observed in the same individuals when 10 ml. volumes of their red cells were sensitized with 20 ml. of anti-D plasma *in vitro* and then reinjected. The intravenous injection of anti-D plasma into an unsensitized D-negative subject had no effect upon his leukocyte levels or temperature, as depicted in the left-hand portion of Figure 7. When, however, type D red cells were then injected about two hours later leukopenia promptly began and was followed by a pyrogenic response. Conversely, as shown in the right-hand part of Figure 7, type D red cells had no discernible effect when injected alone into another unsensitized D-negative subject. However, injection of anti-D plasma about two hours later promptly initiated leukopenia followed later by fever.

In a single experiment, 10 ml. of Cr^{51}-labeled type D red cells was added with sterile precau-

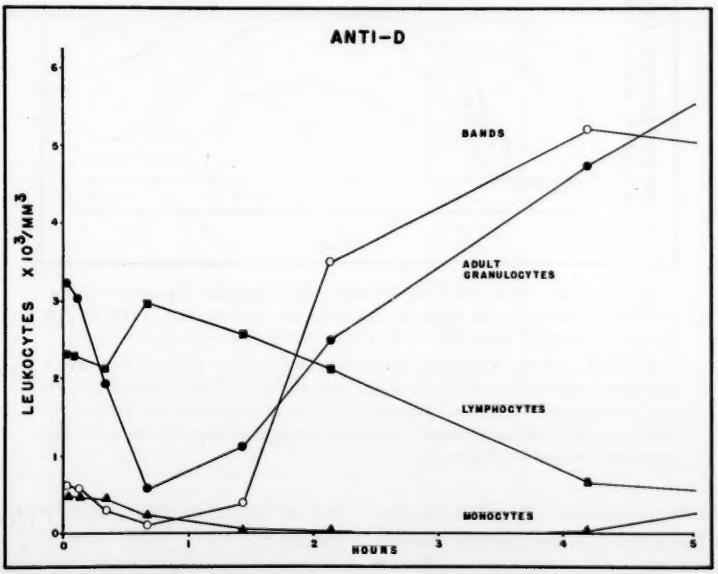

FIG. 5. CHANGES IN LEUKOCYTE DIFFERENTIAL COUNTS FOLLOWING INTRAVENOUS INJECTION OF TYPE D RED CELLS INTO A SENSITIZED D-NEGATIVE SUBJECT

As in ABO-incompatibility, striking reductions in granulocytes and monocytes accompanied red cell sequestration, whereas lymphopenia was delayed until two or more hours afterward. Band forms increased markedly in number, reaching eight times the initial value four hours after injection. As in Figure 2, the recovery of monocyte levels was slower than that of the granulocytes and there was no later monocytosis.

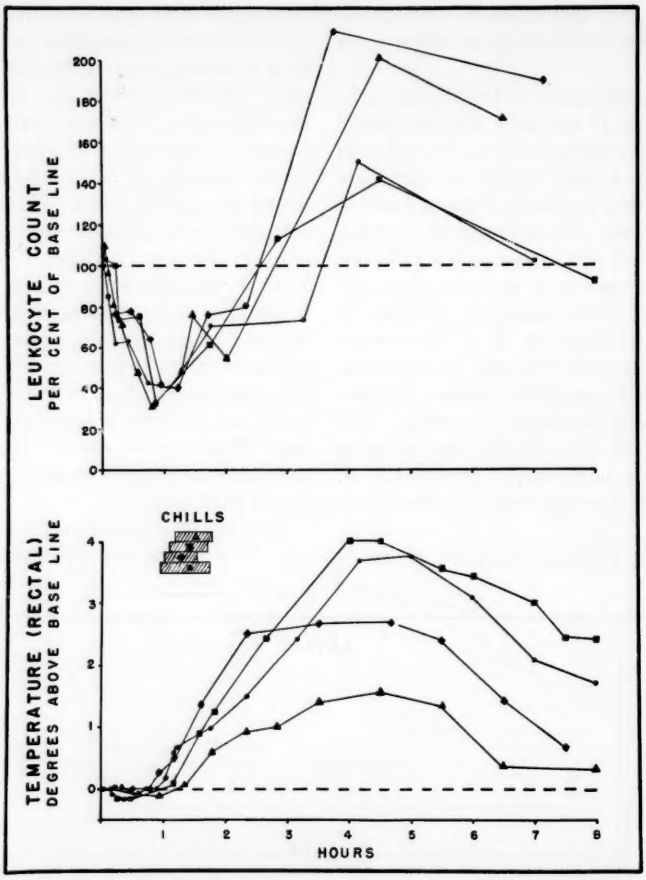

FIG. 6. LEUKOCYTE AND BODY TEMPERATURE CHANGES FOLLOWING INTRAVENOUS INJECTION INTO NORMAL SUBJECTS OF AUTOGENOUS TYPE D RED CELLS PREVIOUSLY SENSITIZED *IN VITRO* WITH ANTI-D

A slowly-evolving leukopenia followed by leukocytosis and a rather severe pyrogenic reaction is evident in each of four subjects. The average leukocyte and temperature responses following injection of sensitized autogenous red cells are very similar to those following injection of type D red cells into sensitized subjects (Figure 4).

tions to a plastic bag containing 500 ml. of freshly-drawn, whole blood from a D-negative subject. To this mixture was added 20 ml. of anti-D plasma and the suspension was incubated at 37° C. for two hours. The entire mixture was then injected into the D-negative subject in a period of six minutes. The subsequent rate of Cr^{51}-labeled red cell sequestration, the leukopenia, and the pyrogenic response were quite similar in extent and in timing to those experiments portrayed in Figure 6.

In both the experiments with ABO-incompatible red cells and the type D red cells exposed to anti-D, the rates at which red cell sequestration occurred were very similar to the rates at which leukopenia developed. In Figure 8, mean values of red cell survival, leukocyte levels, and plasma hemoglobin levels following injection of such

red cell suspensions, are portrayed for ready comparison.

The influence of several agents on the leukopenia and pyrogenic response to incompatible blood was examined. Two subjects were injected with sufficient heparin to prolong the clotting times of their blood in glass tubes to approximately one hour. Thereafter these subjects were injected with ABO-incompatible red cells and anti-D sensitized autogenous red cells, respectively. However, the heparin did not alter the leukopenic response to either injection, or the pyrogenic response to the sensitized cells. One subject was given 1 Gm. of aspirin one hour before the injection of 10 ml. of anti-D sensitized autogenous red cells and 1 Gm. of aspirin every two hours thereafter for six hours. The characteristic pattern of sequestration of the injected red cells, of the plasma hemoglobinemia, and of the leukopenia were unaffected; however, no chills were observed and only a slight rise in rectal temperature appeared during the six hour observation period. Two subjects were given 50 mg. of cortisone orally every six hours for 24 hours and 48 hours, respectively, before the injection of 10 ml. of anti-D sensitized autogenous red cells. In both instances there was an apparent suppression of the usual pyrogenic response, although the rate and site of sequestration of the sensitized red cells, and the subsequent leukopenia, occurred as it had in subjects untreated with cortisone.

Five subjects receiving 10 ml. of ABO-incompatible red cells, and three subjects receiving anti-D sensitized autogenous red cells were observed for alterations in blood platelet and plasma fibrinogen levels. In neither group did platelet or fibrinogen levels fluctuate significantly during the periods of observation.

Effects of red cells altered by nonimmunological mechanisms

Normal red cells were altered *in vitro* under sterile conditions by several kinds of nonimmunologic procedure. Unless otherwise stated below,

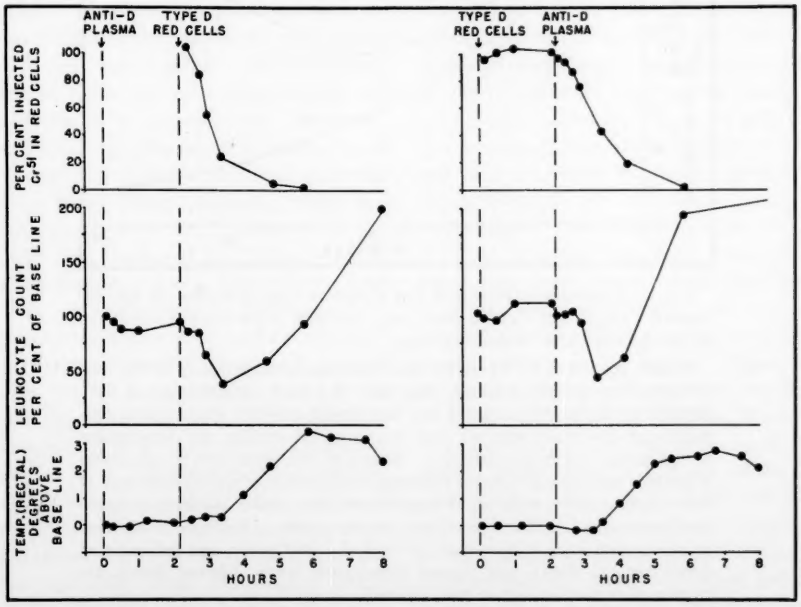

FIG. 7. RED CELL SEQUESTRATION, LEUKOPENIA, AND FEVER ON INTERACTION BETWEEN ANTI-D AND TYPE D RED CELLS IN THE CIRCULATION OF UNSENSITIZED D-NEGATIVE SUBJECTS

Note that leukopenia and fever failed to occur when either anti-D plasma or Cr^{51}-labeled type D red cells were given individually. When, however, both coexisted, the labeled red cells were rapidly removed from the circulation (upper portion), leukopenia developed (middle portion), and fever, initiated by chills, ensued (bottom portion).

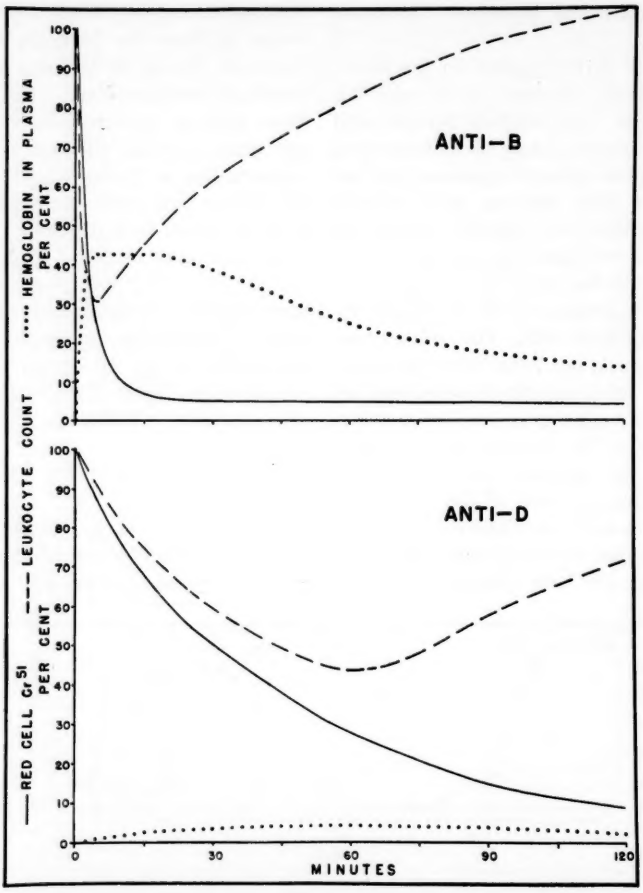

FIG. 8. COMPARISON BETWEEN THE RATES OF SEQUESTRATION OF INCOMPATIBLE TYPE B AND TYPE D RED CELLS AND THE ASSOCIATED APPEARANCE OF LEUKOPENIA AND HEMOGLOBINEMIA

In each portion of the figure the lines represent average values of the data obtained from several different subjects. The rapid sequestration of Cr^{51}-labeled type B red cells injected into five normal subjects whose serum contained anti-B (upper portion) was promptly followed by the development of leukopenia and hemoglobinemia. Similarly, the more slowly developing leukopenia and hemoglobinemia following the injection of Cr^{51}-labeled type D red cells into four sensitized, D-negative subjects (lower portion) occurred simultaneously with the sequestration of these cells. Leukocyte levels (interrupted lines) are expressed as per cent of initial levels, and red cell Cr^{51} (uninterrupted lines) and plasma hemoglobin levels (dotted lines) are expressed as the per cent of that injected.

the cells were labeled with Cr^{51} after the alteration was produced. After reinjection of the altered, Cr^{51}-labeled red cells into their normal donors, these subjects were studied as usual in terms of Cr^{51}-labeled red cell survival, plasma levels of hemoglobin and of Cr^{51}, body distribution of Cr^{51}, leukocyte levels, and body temperature. The results are summarized in Table II.

TABLE II

Observations on the destruction of red cells altered by nonimmune mechanisms

Method of producing red cell alteration	Survival *in vivo* of altered red cells		Hemoglobinemia (>4 mg. %)	Site of sequestration	Systemic effects	
	% sequestered	Half-survival			Fever	Leukopenia
Sterile incubation at 37° C.						
4 hrs. (ACD)*	0		0		0	0
24 hrs. (ACD)	30% (in 20 min.)		0	Liver	0	0
48 hrs. (ACD)	100% (in 40 min.)	4 min.	0	Liver	0	0
48 hrs. (defibrinated)	100% (in 60 min.)	6 min.	0	Liver	0	0
Acetylphenylhydrazine	100% (in 5 days)	8 hrs.	0	Spleen	0	0
Lecithin	68% (in 60 min.)	22 min.	0	Spleen	0	0

* Acid-citrate-dextrose.

A. Sterile incubation. Fifty ml. of normal whole blood mixed with ACD solution was stored at 37° C. for four hours without agitation. The Cr^{51} survival of the derived red cells on subsequent reinjection into the donor was unaffected, and there were no systemic reactions. Fifty ml. of normal whole blood incubated for 24 hours under these conditions showed no visual evidence of autohemolysis. There was a moderate symmetrical shift to the left in the osmotic fragility curve. The P. V. P. test was negative. On reinjection, 30 per cent of these red cells were rapidly sequestered, chiefly by the subject's liver. However, there was no rise in plasma hemoglobin or of plasma Cr^{51}, and no systemic reactions occurred. Normal whole blood (50 ml. amounts) which had been either mixed with ACD solution or defibrinated and then incubated for 48 hours under the conditions described above showed very little autohemolysis. There were moderate increases in methemoglobin concentration, in mechanical fragility, and the most osmotically fragile cells became more fragile, the least fragile cells became less fragile, while the mean osmotic fragility was relatively unchanged. The P. V. P. test was negative. In each instance these cells were abruptly removed from the circulation after their injection, and were sequestered largely in the liver; half-survival times were four minutes for red cells in ACD and six minutes for red cells from defibrinated blood. One of these two studies is presented in Figure 9. Despite the rapidity and completeness of this sequestration, there was no hemoglobinemia (all levels were below 3.5 mg. per cent), no Cr^{51} appeared in the plasma, and there were no febrile reactions or leukopenia. That the injected red cells were actually hemolyzed at the site of their hepatic sequestration, however, was suggested by a rise in indirect-reacting bilirubin, which began about one hour after injection.

B. Acetylphenylhydrazine. Normal red cells which had been labeled beforehand with Cr^{51} were exposed to a sterile solution of 1 per cent acetylphenylhydrazine for one hour at 37° C., and then washed once prior to reinjection into the donor. Although there was no supernatant hemoglobin in this preparation, much of the intracorpuscular hemoglobin had been precipitated as Heinz bodies (an average of four Heinz bodies per red cell) or had been converted to methemoglobin and sulfhemoglobin (54 per cent and 4 per cent of the unprecipitated hemoglobin, respectively). The P. V. P. test was negative. These red cells on reinjection were removed from the blood stream at a gradually diminishing rate, with a half-survival time of eight hours, and were sequestered almost entirely in the spleen. Again no hemoglobin or Cr^{51} was released into the plasma, and no febrile or leukopenic response occurred.

C. Lecithin. The red cells from 35 ml. of normal whole blood in 12.5 ml. of ACD solution were labeled with Cr^{51}, washed with physiologic saline, and then suspended in a 1 per cent sterile solution of animal lecithin at 25° C. for one hour. After rewashing, these lecithinized red cells were spherocytic in appearance under the microscope and their osmotic fragility was found to be symmetrically "shifted to the left," with the mean cellular fragility increased from 0.41 Gm. per cent sodium chloride before treatment with lecithin to

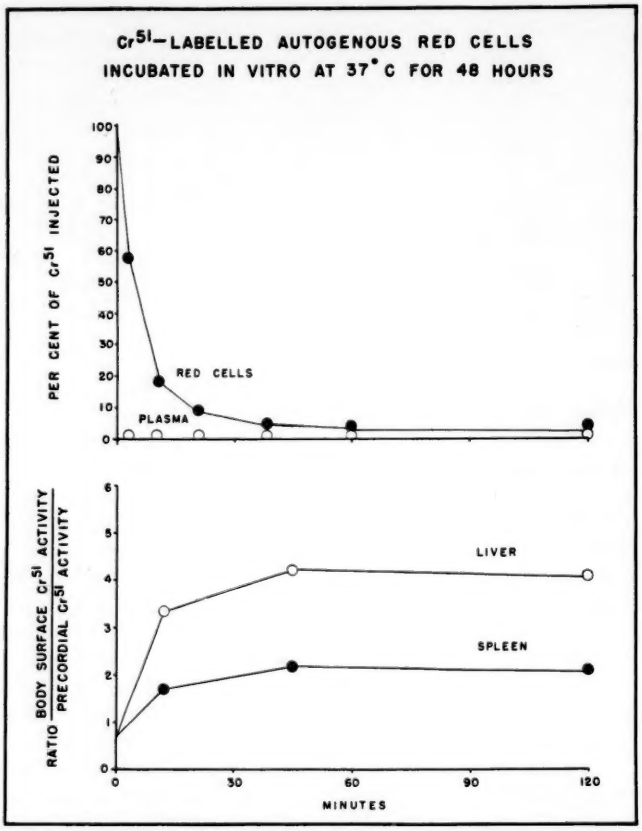

FIG. 9. THE RAPID HEPATIC SEQUESTRATION OF RED CELLS AFTER THEIR PROLONGED INCUBATION AS STERILE DEFIBRINATED BLOOD *IN VITRO*

Despite the extreme rapidity of red cell sequestration by the liver, comparable in rate and site to that seen with ABO-incompatibility, there was no hemoglobinemia. That an elution *in vivo* of the Cr^{51}-label did not ensue is indicated by the failure of Cr^{51} to appear in the subject's plasma. There was no change in blood leukocyte levels or in body temperature of either subject who received red cells altered by incubation *in vitro* for 48 hours.

0.62 Gm. per cent afterwards. Lecithinization increased the mean corpuscular volume from 86 to 103 μ^3 and the mean corpuscular hemoglobin concentration was decreased from 31 to 28 per cent. On reinjection into the donor, about two-thirds of the lecithinized spherocytes were sequestered in the spleen (half-survival, 22 minutes). Thereafter circulating red cell Cr^{51} slowly increased from 32 per cent of the injected dose in one hour to 57 per cent of the injected dose at the end of 24 hours (declining at a normal rate thereafter) while splenic radioactivity behaved in a reciprocal fashion (Figure 10). During the acute and, in part, transient [7] sequestration of lecithinized spherocytes by the patient's spleen, there was no rise in plasma

[7] Although one generally assumes that splenic sequestration of red cells causes their injury or destruction, it appears that these transiently trapped spherocytes were in some manner rejuvenated. Incubation for eight hours at 37° C. *in vitro* of lecithinized red cells in normal heparinized plasma failed to improve their osmotic fragility.

hemoglobin or Cr^{51}, and there were no changes in the patient's leukocyte levels or body temperature.

Observations in vitro and in the tourniquet-obstructed arm

The effects of red cell isoantibody interactions on leukocytes *in vitro* were studied by incubating various combinations of red cells, compatible and incompatible plasmas, and leukocytes. Leukocyte-rich suspensions were obtained by first removing red cells from whole blood by light centrifugation and then gently spinning down the leukocytes, as well as some of the platelets, from the supernatant plasma. Siliconized glassware was employed throughout and experiments were conducted in various systems involving plasma containing heparin or EDTA, plasma obtained by

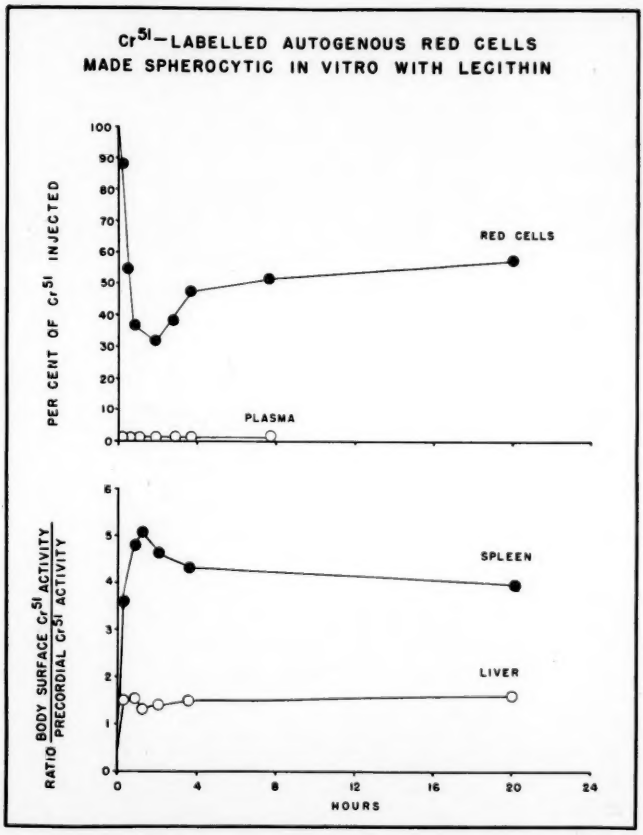

FIG. 10. SPLENIC SEQUESTRATION OF LECITHINIZED SPHEROCYTES

The rate of sequestration of these spherocytes resembled that of red cells coated with incomplete antibodies (2). However, the apparent "escape" from the spleen of a portion of these red cells and the complete absence of hemoglobinemia were never observed after comparable injections of anti-D sensitized red cells. Note that many of the cells were detained in the spleen for three or four hours, and some for as long as 22 hours. Thereafter (not shown) red cell Cr^{51} levels declined slowly in a normal fashion. Although in this experiment approximately 7 or 8 ml. of red cells was sequestered in the spleen, there was no accompanying leukopenia or febrile reaction.

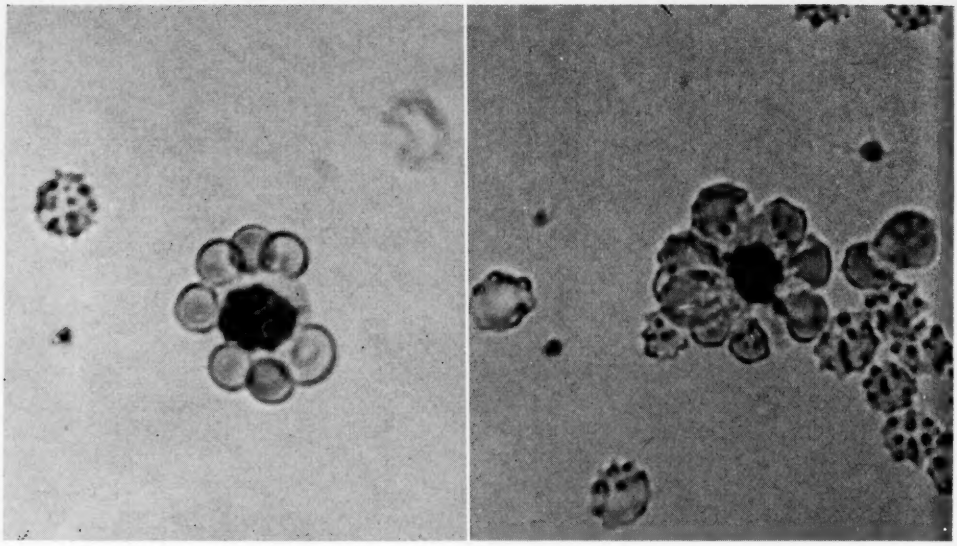

FIG. 11. ROSETTE-TYPE AGGLUTINATES INVOLVING ANTI-D SENSITIZED RED CELLS AND GRANULOCYTES FROM A D-SENSITIZED SUBJECT

Following incubation at 37° C. for 30 minutes of type D red cells suspended in resin-decalcified incomplete anti-D plasma to which freshly-obtained leukocytes from a D-sensitized donor had been added, there was no red cell agglutination. However, red cell clumping around leukocytes (usually granulocytes) was apparent as shown. The preparation was diluted with saline (thus causing some red cell crenation), stained supravitally with neutral red, and photographed under a cover slip. Note the smooth, spherocytic contour of the adherent red cells, particularly on the left, the "invasive" appearance of the granulocyte processes, particularly on the right, and the lack of involvement of platelets.

passage through a cation-exchange resin column, and serum from defibrinated blood,[8] respectively.

In such systems containing type A red cells and type O plasma and leukocytes, immediate red cell agglutination was microscopically visible. At the periphery of many of the clumps one or more leukocytes (invariably granulocytes or monocytes) were seen to be adherent. Red cells contiguous to these leukocytes underwent sphering within a few minutes, and shortly thereafter erythrophagocytosis frequently became evident. Platelets were also seen to adhere to these leukocytes, but no more than to "free" leukocytes. Apart from these mixed agglutinates, clumping of leukocytes or of platelets did not occur to a greater extent than in control mixtures in which red cells were suspended in compatible plasma. The total numbers of leukocytes and of platelets were unchanged during the period of observation.

In systems involving type D red cells and plasma (containing incomplete anti-D) and leukocytes from D-sensitized subjects, no red cell agglutination occurred. Within a few minutes of admixture, however, the antibody-sensitized red cells were seen to cluster around individual leukocytes, often producing rosette-patterns (Figure 11). These red cells also became spherocytic. Erythrophagocytosis occurred to only a small extent, and then only after an hour or two. Occasionally two or more leukocytes were involved in mixed agglutinates. Platelets were not involved in these mixed clumps, and there was no evidence of pure

[8] Whereas defibrinated blood is to be preferred to plasma containing anticoagulants when the action of complement is under study, it is less satisfactory for harvesting leukocytes. We have observed that in the course of defibrinating whole blood with glass beads, a sharp reduction of leukocyte numbers occurs within a minute of the onset of fibrin-deposition. This reduction in leukocytes results almost entirely from adherence of granulocytes to the clot.

leukocyte or platelet agglutination. The total leukocyte and platelet counts were unchanged. When mixtures were made of type D red cells, plasma containing anti-D, and leukocytes from unsensitized subjects, adherence of red cells to leukocytes occurred more slowly and to a lesser extent; in such suspensions mixed agglutination became prominent only when the cells were lightly centrifuged together.

In an effort to circumvent the possible artificialities of *in vitro* preparations, several observations were carried out within the vascular bed of the tourniquet-obstructed forearms of normal human subjects. In these studies the subjects were given 50 mg. of heparin intravenously, and Cournand needles were placed in the antecubital veins of each arm. A blood pressure cuff was then inflated around one arm at a pressure midway between systolic and diastolic blood pressures, and immediately thereafter a small volume of Cr^{51}-labeled red cells was injected into the brachial artery just below the inflated cuff. Small specimens of venous blood were withdrawn into heparin at frequent intervals through each Cournand needle before and after deflation of the cuff, and were examined for radioactivity, for leukocyte levels, and, in blood suspended in warm saline, for autoagglutination.

In each of two observations approximately 2 ml. of Cr^{51}-labeled type A-incompatible red cells was injected intra-arterially into the tourniquet-obstructed forearm. As portrayed in Figure 12, maximal numbers of labeled red cells soon appeared in the local venous samples, but such radioactivity then slowly fell, presumably as a result of the escape of venous blood past the cuff and of venous flow through the humerus. In the first venous samples containing labeled red cells, red cell agglutinates were visible. Often leukocytes were attached to the periphery or occasionally

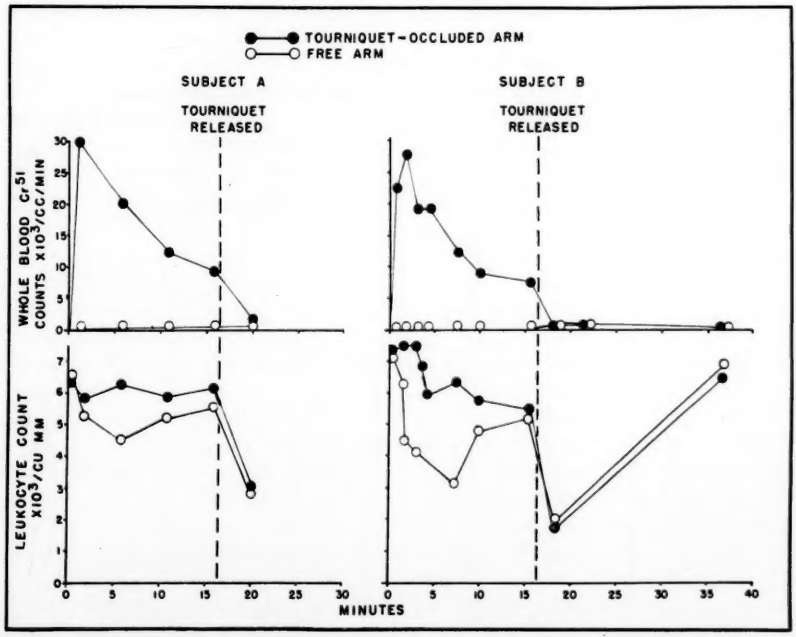

FIG. 12. THE EFFECT ON LEUKOCYTE LEVELS OF Cr^{51}-LABELED INCOMPATIBLE RED CELLS INJECTED INTRA-ARTERIALLY INTO THE TOURNIQUET-OCCLUDED FOREARMS OF NORMAL SUBJECTS

Results are of observations made in two subjects. Note that despite active local red cell agglutination and hemolysis, leukolysis in the "tourniquet-isolated" arm did not occur. However, the systemic effects of immune red cell agglutination are apparent in the systemic leukopenia that promptly followed the release of the tourniquet.

were present in the center of these agglutinates; and, as *in vitro,* the red cells adjacent to these leukocytes appeared to be spherocytes. Nevertheless, there was little or no reduction in leukocyte levels in the venous blood samples from the "obstructed" arms, while venous blood in the opposite, "free" arm, which registered the presence of only traces of Cr^{51}-labeled red cells, showed transient leukopenia, presumably as an effect of those injected red cells which escaped from the obstructed arm. Meanwhile, in venous samples from the obstructed arm progressive hemoconcentration developed and the plasma hemoglobin level rose to over 100 mg. per cent in each observation, although no hemoglobin was detected in plasma from the opposite forearm. In each observation, on deflation of the cuff the Cr^{51} activity of the red cells and the leukocyte level both abruptly fell in venous blood from the obstructed arm. Subsequent body surface scanning revealed a heavy accumulation of Cr^{51} in the subjects' livers, and to a lesser extent in their spleens, but no retention of Cr^{51} activity was detectible in the subjects' previously obstructed forearms.

In a single comparable observation, in which 2 ml. of Cr^{51}-labeled, anti-D sensitized autogenous red cells was injected intra-arterially into the obstructed forearm of a normal subject, there was no effect on leukocyte levels in either arm during a 15 minute period of venous stagnation, and no mixed agglutinates were seen in local venous samples.

Observations with incompatible red cells injected intradermally

In order to examine the possibility that cells other than leukocytes may be injured by the reactions between red cells and their antibodies, compatible and incompatible red cells were injected intradermally into several subjects. Generally 0.1 ml. of a 50 per cent saline suspension of washed red cells was injected intradermally into the flexor surface of the forearm: test cells at one site and autogenous or compatible cells at another.

TABLE III

Relation between the dermal response to ABO-incompatible red cells and isoantibody titers

Subject	Injected red cell type	Subject's red cell type	Subject's isoantibody titer*		Skin response (wheal and erythema)
			Hemolysin	Agglutinin	
			Positive skin responses		
JA	B	A	4	128	4+
WI	A	O	4	128	4+
PU	A	O	4	32	4+
VA	B	A	1	64	3+
OR	A	B	1	32	2+
WA	A	O	1	64	2+
WA	B	O	Trace	64	1+
WL	A	O	Trace	64	2+
DA	A	O	0	128	1+
			Negative skin responses		
WI	B	O	0	32	0
PU	B	O	0	32	0
WL	B	O	0	16	0
DA	B	O	0	8	0
O'L	A	O	0	32	0
O'L	B	O	0	128	0
ME	B	O	0	64	0
HO	B	A	0	8	0
GO	A	O	0	32	0
GO	B	O	0	8	0
VI	A	O	0	64	0
VI	B	O	0	16	0
MC	A	O	0	64	0
MC	B	O	0	8	0
PH	A	O	0	64	0
PH	B	O	0	64	0

* Measured by incubating equal volumes of various dilutions of fresh serum and 5 per cent washed red cells in saline for one hour at 37° C. The titer is expressed as a reciprocal of the minimally effective serum concentration.

Most subjects injected with 0.1 ml. of ABO-incompatible red cells manifested no immediate or delayed skin reactions and the progress of visible pigment breakdown proceeded at the same rate as in the sites of injection of autogenous red cells. However, approximately one-third of normal subjects developed a wheal at the site of injection of incompatible red cells (Table III and Figure 13). Generally wheal formation became apparent within 2 to 5 minutes of injection, the swelling was maximal in 10 to 20 minutes, and a diminution of swelling was noticeable in about one hour. A surrounding erythema and local venous constriction were usually evident simultaneously during the onset of wheal formation. Two subjects noted transient itching during wheal formation, and occasionally during the ensuing 24 to 48 hours. As noted in Table III, those subjects who developed urticaria at the site of injection of incompatible red cells usually possessed hemolysins for those cells. None of the subjects with isohemolysins demonstrable by conventional techniques *in vitro* failed to develop an acute wheal in response to intradermal incompatible cells. Only one subject not manifesting isohemolysins *in vitro* exhibited dermal sensitivity to incompatible red cells. Ten type O subjects were injected intradermally at different sites with type A and with type B cells. In the three subjects with isohemolysins for type A red cells, but not for type B cells, there were positive skin reactions to type A cells only.

Two type A subjects who were injected intradermally with 0.1 ml. of type O serum which hemloyzed type A cells showed small, questionable wheals and no erythema at the injection sites. However, when the same type O serum was mixed with the subjects' type A cells *in vitro,* and 0.1 ml. of the mixture was injected, a very strong wheal-and-erythema response occurred (Figure 13).

The injection of 0.1 ml. of a 50 per cent suspension in saline of type D red cells into two otherwise normal D-negative subjects sensitized to the D antigen caused no immediate or delayed skin reactions. Similarly the intradermal injection into two subjects of type D red cells sensitized *in vitro* with anti-D serum produced no skin reactions. That wheal formation was not caused by the injection of red cell agglutinates *per se* was demonstrated by injecting 0.1 ml. of washed autogenous red cells which had been strongly agglutinated by ferric chloride. There was no dermal reaction.

FIG. 13. IMMEDIATE SKIN REACTIONS PROVOKED BY ISOHEMOLYSIS

Three injections were given intradermally within a total period of 4 or 5 minutes and this photograph was taken 40 minutes later. The injection into this type A subject of 0.1 ml. of a 50 per cent suspension of type O red cells in saline caused no reaction (site A). The injection of a comparable preparation of type B red cells, however, caused an immediate wheal and erythema response (site B). A similar, even more intense wheal and erythema response followed the injection of site C of a mixture of autogenous type A red cells and type O serum containing isohemolysins. Injections of autogenous serum alone (not shown) left little or no swelling. Injections of serum containing isohemolysins against the subject's red cells (not shown) provoked a slight wheal without erythema.

DISCUSSION

Leukopenia during immune hemolysis

The observations presented indicate that leukopenia is a characteristic feature of immune hemolysis *in vivo,* whether this hemolysis arises from hemolysins or from complete or incomplete agglutinins. The leukopenia, initially at least, reflects a fall in granulocyte and monocyte levels. The leukopenic response is generally proportional to the maturity of these cells, thus being striking with respect to adult neutrophils, moderate with band forms, and absent with metamyelocytes.

The leukopenia is clearly not a consequence of the direct action of antibodies on leukocytes since it is most readily produced in subjects by the injection of washed incompatible red cells. It is also clear that the leukopenia is not simply the result of antigen-antibody interaction, since the intravenous

injection of anti-A serum into type A recipients and of anti-D serum into type D recipients caused little or no leukopenia, unless actual hemolysis was produced. That leukopenia appears to reflect some feature of the immune lysis of red cells rather than the mere coating of the cell with antibodies or the hemolysis *per se* is indicated by the following observations: *1*) Leukopenia occurred slowly with the slowly-sequestered and -hemolyzed anti-D sensitized red cells, and rapidly with the rapidly-sequestered and -hemolyzed ABO-incompatible cells. *2*) When antiserum was injected intravenously in amounts insufficient to cause hemolysis *in vivo* no leukopenia ensued, whereas when the same volume of antiserum was mixed with the recipients' incompatible red cells prior to injection, resulting in the hemolysis of these cells *in vivo,* leukopenia occurred. *3*) The injection of whole red cell hemolysates prepared by freezing and thawing or of osmotically released hemoglobin had no effect on circulating leukocytes nor did the brisk sequestration and destruction of red cells *in vivo* by several nonimmunologic mechanisms, including preliminary alteration of the injected red cells by prolonged incubation or by exposure to lecithin or acetylphenylhydrazine. It is also evident that a response by leukocytes to foreign red cells as such does not underlie the leukopenia, since anti-D coated autogenous red cells were active in this respect; furthermore, a patient with acquired hypogammaglobulinemia, who was injected with ABO-incompatible red cells and who failed to destroy these cells rapidly (*2*) showed no leukopenic response to the incompatible cells.

The finding that leukopenia followed the injection of soluble A substance into type O individuals, but not into type A individuals, indicates, nevertheless, that leukopenia may result from antigen-antibody interaction when these are in a noncellular suspension or solution. Indeed it is well established that the injection into sensitized animals of noncellular antigens provokes leukopenia (20–23) and often thrombocytopenia (24) much as does the injection of bacterial extracts ("pyrogens" or "endotoxins") into normal animals, including man (25–30).

The observations *in vitro,* as well as in the tourniquet-obstructed forearm, indicate that leukocytes, particularly granulocytes and monocytes, tend to adhere strongly to antibody-coated red cells. In systems containing ABO-incompatible serum and red cells our observations are similar to those on the action of complement-fixing dog and human isoantibodies by Bakemeier and Swisher (12) who described the adherence *in vitro* of leukocytes to red cell agglutinates in the absence of hemolysis, and the formation of heavy mixed agglutinates of red cells and leukocytes when hemolysis was present. Butler (31) has described clearly the evolution *in vitro* of mixed agglutinates due to ABO-antibody-coated red cells, showing that the leukocytes were attached in common to the red cells rather than to each other. Neither *in vitro* nor *in vivo* was there evidence of direct leukolysis or of pure leukoagglutination. In our hands a tendency for leukocytes to adhere to antibody-coated red cells was also evident in the absence of complement fixation or of red cell agglutination. Thus, anti-D sensitized red cells clustered about, and adhered to, leukocytes (particularly those of D-sensitized subjects) to form rosettes, as in Figure 11. In general the tendency for leukocytes to participate in mixed agglutinates varied directly with the speed with which the involved antibody caused hemolysis *in vivo,* and the speed and extent with which erythrophagocytosis occurred *in vitro.*

Although a tendency exists for contiguous leukocytes to adhere to antibody-coated red cells, it is unlikely that the leukopenia observed *in vivo* results directly from a filtration from the circulation by certain capillary beds of red cell-leukocyte clumps or of erythrophagocytic leukocytes. The improbability of such a mechanism may be inferred from the facts that leukopenia follows the injection of soluble A substance and that leukocytes may be sequestered within a few minutes of the injection of incompatible red cells in a ratio as great as 15 leukocytes to 1 red cell (Table I). It is more likely that the leukopenia results from a mechanism common to all intravascular antigen-antibody reactions, particulate or nonparticulate, and is akin to the physiologic response to bacterial endotoxins.

The conclusion most consistent with the observations summarized above is that the reaction of antibody with antigen on the red cell surface causes a change in the surface of nearby leukocytes, and that this leads to mutual adhesion and

often to actual phagocytosis. However, striking leukopenia occurs only when hemolysis of the sensitized red cells, either by plasma or by tissue factors, releases (in comparative profusion) the antigen-antibody complexes into the bloodstream. This presumably is what occurs without the vehicle of the red cells when nonparticulate antigens or bacterial endotoxins are injected intravenously.

The general nature of the change in the leukocyte surfaces which attends the interaction between antigens and antibodies, and, as well, the action of bacterial endotoxins, is an increase in the "stickiness" of leukocytes, thus disposing them to adhere to one another and probably to vascular endothelium everywhere (*22*, 32–34). Presumably filtration and adhesion of such leukocytes would be most prominent in those organs with large or effective filtering beds, namely the lungs, liver and spleen. Indeed, there have been numerous reports of cramming of the pulmonary vasculature with leukocytes during anaphylaxis (*26*, *27*, 35).

The leukocytes most affected in this manner by immune reactions are the granulocytes and monocytes. In the present study eosinophils and probably basophils also were so affected, whereas lymphocytes were conspicuously spared. The lymphopenia which occurred about two hours after the injection of red cells, particularly when anti-D sensitization was involved, in all likelihood reflects an adrenocortical effect. Our failure to observe the thrombocytopenia after the injection of incompatible red cells described by others as a result of incompatible transfusions (11, 36) and said to be associated with platelet-red cell mixed agglutinates (11, 36), may reflect the smaller quantity of incompatible red cells injected by us and the smaller amount of complement fixation involved. Indeed, thrombocytopenia is known to accompany anaphylaxis in certain instances (24), and possibly the susceptibility of platelets to injury by antigen-antibody reactions is analogous to, but less than, that of leukocytes.

The actual fate of the leukocytes which disappeared from the circulation in this study, as in similar studies in general, is not entirely clear. It is possible that many of these cells were only transiently sequestered or that they temporarily emigrated from the vasculature (32) and returned within a few hours to the general circulation. However, it is clear that the initial recovery of leukocyte levels was due not to the return of the sequestered leukocytes, but in large part, at least, to the appearance of young cells including bands, metamyelocytes, myelocytes, and some immature monocytes. This outpouring of young leukocytes was evident within less than an hour after the injection of ABO-incompatible red cells, whereas in dogs subjected to mechanical leukophoresis, Craddock, Perry, and Lawrence (*37*) have found that a delay of about two hours generally precedes the initial rise in leukocyte levels. This delay is believed to reflect a "priming" of the leukocyte-depleted vasculature. Their finding that the immature leukocytes which appear during the leukocytosis are derived from the marrow pool, whether the preceding leukopenia was the result of leukophoresis (*37*) or of the injection of typhoid vaccine (38), almost certainly applies here.

The possible physiological utility of the extraordinary leukocyte responses to immune hemolysis is conjectural. Evidence for the participation of circulating leukocytes in the phagocytic destruction of antibody-sensitized red cells *in vivo* is largely limited to immune systems involving complement fixation. In its absence there is usually meager evidence of erythrophagocytosis *in vivo;* and erythrophagocytosis *in vitro* of red cells sensitized with anti-D serum or of red cells from most patients with acquired hemolytic anemia is comparatively slow to appear and limited in extent. Nevertheless, the studies *in vitro* of Zinkham and Diamond (39), Butler (31), and Bakemeier and Swisher (12), as well as those described here, indicate that antibody-sensitized red cells, and the "sensitized" red cells of acquired hemolytic anemia, whether or not complement is involved, tend in various degrees to cling to granulocytes and monocytes. This phenomenon consists initially of red cell adherence to contiguous leukocytes, associated with apparent "sphering" of the involved red cells; actual phagocytosis of the trapped red cell may then supervene, particularly if complement is fixed. Of course, for sensitized red cells to be destroyed by fixed or circulating leukocytes it may not be essential that actual erythrophagocytosis occurs. Turner (40) has reported that leukocytes may act as cofactors in the lysis by cobra venom of the red cells of certain animal species by providing a source of lecithin which is rendered hemolytic by the venom

lecithinase. Nevertheless, we did not succeed in making analogous observations by incubating together various combinations of leukocytes, lecithin, fresh serum and normal or anti-D sensitized red cells. In general, however, it seems plausible that, by virtue of the phenomena described above, the circulating leukocytes may aid the fixed macrophages of the reticuloendothelial system in destroying trapped, antibody-sensitized red cells. Jointly their function may be considered that of cleaning the filter beds of the spleen, liver and lungs that are mechanically responsible for the initial sequestration of the red cells.

That anti-D sensitized red cells adherent to leukocyte surfaces appear spherocytic is evident in Figure 11. Such spherocytosis is also apparent in photographs of the fixed smears of Zinkham and Diamond (39), which show sensitized red cells of acquired hemolytic anemia patients clustered around leukocytes prior to erythrophagocytosis. A mysterious feature of immune hemolysis has been that the action of antibodies on red cells *in vivo* leads to spherocytosis, whereas this is not so when such antibodies are incubated with red cells *in vitro* (1). Similarly spherocytosis is a common feature of the peripheral blood of patients with acquired hemolytic anemia (41), whereas incubation of normal red cells with the serum of these patients does not cause spherocytosis even when sensitization or agglutination is provoked. It is tempting to conclude that the spherocytosis observed *in vivo* during immune hemolysis and in patients with acquired hemolytic anemia is a consequence of the surface effects of leukocytes and of tissue macrophages upon antibody-coated red cells. Thus far our efforts to demonstrate *in vitro* changes in the osmotic fragility of antibody-sensitized red cells in the presence of leukocyte suspensions have perhaps been frustrated by the comparatively small number of red cells involved in the mixed agglutinates which develop.

Fever during immune hemolysis

Fever and chills often accompany severe hemolytic episodes, such as attend transfusions of incompatible homologous or heterologous blood. However, the mechanism of this pyrogenic response has been obscure. In their search for tissue pyrogens, Bennett and Beeson (42) found that red cells hemolyzed *in vitro* by various nonimmunologic procedures failed to provoke fever on injection into normal rabbits, whereas the injection of dog serum containing a high titer of agglutinins against rabbit red cells, in distinction to dog serum inactive against rabbit red cells, did produce a febrile response. That the presence of a foreign protein or tissue need not underlie the fever of such hemolysis, however, is evident, in that patients with paroxysmal cold hemoglobinuria develop comparable pyrogenic responses following hemolytic paroxysms induced by cooling of an extremity (7, 8).

The findings reported here as to the effects of immune hemolysis in man on body temperature may be summarized as follows: *1*) Hemolysis by isoantibodies in man was generally attended by a febrile response. *2*) Hemolysis of comparable degree by nonimmunologic mechanisms did not cause fever. *3*) The slow destruction *in vivo* of red cells by incomplete antibodies (*i.e.*, anti-D) produced decidedly more striking febrile responses than did the rapid destruction of comparable amounts of red cells by complete agglutinins or hemolysins (*i.e.*, anti-A and anti-B), although the latter agents induced much more hemoglobinemia. *4*) A febrile response accompanied immune hemolysis regardless of whether the interacting red cells or antibodies, or neither, were native to the subject. *5*) The pyrogenic response was invariably accompanied by leukopenia, and the onset of fever followed approximately one hour after the injection of antigenic cells or antibody. *6*) Finally, as with the leukopenia, fever did not occur when red cells and antibody interacted *in vivo* unless red cell sequestration or hemolysis was also produced.

An interesting association between the rate of hemolysis and the occurrence of fever emerged from the studies summarized above. The rapid sequestration of red cells by anti-A or anti-B was associated with a rapid, transient leukopenia and with only slight fever. The slower sequestration of red cells by anti-D was accompanied by a more slowly developing and more prolonged leukopenia and with marked febrile responses.[9] These two

[9] It is presumed that the rather striking differences observed in these studies between the effects of these two immune systems are in part a function of the number of red cells involved and that these differences may be obscured when the mechanisms of immune destruction are saturated by the comparatively large amounts of red cells generally involved in transfusions of incompatible blood.

patterns of response, respectively, to ABO-incompatibility and to D-incompatibility resemble those which occur when bacterial pyrogens are injected into "tolerant" rabbits and into normal rabbits, respectively. Tolerance to injections of pyrogens in rabbits is characterized by an accelerated removal of the pyrogenic material from the bloodstream (43–45) and thereafter a diminished febrile response. This pattern may be likened to that reported here with ABO-incompatibility. In contrast, pyrogens injected into normal rabbits are removed comparatively slowly from the blood stream and fever is pronounced (43–45), as is the case with the slow immune destruction of type D red cells. It may be postulated that the slow removal of bacterial products or of antigenic materials is attended by more widespread tissue alterations than is possible when abrupt removal of the material occurs. It is alternatively conceivable that such slow rates of particle-removal reflect simply the fact that the spleen is providing the chief site of particle-sequestration, and that in the spleen special conditions may exist favoring the alteration of crucial cell types (*i.e.*, leukocytes, platelets, reticuloendothelial cells, lymphatic tissue). In support of such a possibility was the observation (not reported above) that the injection of Cr^{51}-labeled, anti-D sensitized red cells into two splenectomized subjects was followed by a comparatively slow sequestration of the sensitized red cells by the subjects' livers (2). The usual strong febrile response one hour after injection did not appear but, rather, a slight prolonged temperature elevation of less than 1° F. It is conceivable in view of such considerations that the severity of the febrile paroxysms in malaria depends upon the splenic sequestration of parasitized red cells. That the spleen, possibly for similar reasons, plays a unique role in the formation of antibodies to particulate antigens is indicated by the fact that splenectomized human (46) or animal (47) subjects form antibodies in response to intravenously injected heterologous red cells much less readily than do normal subjects, whereas the antibody response of splenectomized animals to particulate antigens when injected subcutaneously is unimpaired (47). Also it seems likely that the natural (48) or experimental (49) sensitization of D-negative subjects occurs less readily with ABO-incompatible type D red cells than with ABO-compatible type D red cells because ABO-incompatible cells are abruptly removed from the circulation after injection, largely in the liver, and are afforded comparatively little chance for contact with specially-disposed lymphatic tissue, such as that of the spleen.

There is still uncertainty as to whether the febrile response to exogenous bacterial pyrogens is ordinarily mediated through the release of an endogenous pyrogen (42, 44), or results from a direct effect of exogenous pyrogen on the central nervous system (50), or both. However, there is much evidence that endogenous pyrogens exist in granulocytes (42), and that in man the direct interaction of bacterial pyrogens and leukocytes *in vitro* leads to the liberation of rapidly-acting pyrogenic material, as shown by Cranston, Goodale, Snell, and Wendt (51, 52). It is therefore of interest that when anti-D sensitized red cells were incubated *in vitro* with an even larger quantity of leukocytes than was employed by Cranston and co-workers, there was no hastening of the pyrogenic effect. Actual red cell lysis by the immune mechanism appears to be required for the febrile response as well as for the leukopenic response. The reinjection of soluble bacterial (23) and nonbacterial (53) antigens has been shown to produce fever as well as leukopenia (20–23). Therefore the association of both fever and leukopenia with actual lysis by immune mechanisms suggests that both reactions are caused by the release into the blood stream of antigen-antibody complexes from hemolyzing red cells.

Dermal responses to immune hemolysis

With one exception, incompatible red cells injected intradermally caused wheal and erythema reactions only when some degree of lysis of the red cells by the subject's serum was demonstrable *in vitro*. Likewise, intradermal injection of autogenous red cells after incubation with hemolytic (anti-A) sera *in vitro* caused similar responses. These observations suggest that immune lysis by complement fixation provoked the dermal reaction. The wheals resembled those produced by the injection of histamine or of allergens into sensitive subjects. The pronounced constriction of nearby veins which appeared after the injection of ABO-incompatible red cells into appropriate subjects also suggested the action of 5-hydroxytryptamine

(serotonin) (54). Serotonin has been shown to cause edema in animals and to account for much of the edema provoked by foreign proteins (55). Furthermore, it has been shown that serotonin (56), as well as histamine (57), is released when antigen-antibody reactions occur in the presence of blood cells *in vitro*, and that in the case of serotonin, platelets appear to be the cellular source and complement is apparently involved (56). In rabbits, anaphylaxis is associated with immediate rises in plasma levels of serotonin and histamine, and concomitant falls have been recorded in the levels of these substances in blood cells (58).

In order to determine whether serotonin produced dermal effects resembling those of red cells undergoing immune lysis, two normal subjects were injected intradermally with 0.1 ml. volumes of saline containing various amounts of serotonin. In these two subjects 0.1 μg. of serotonin creatinine sulfate [10] produced a doubtful, and a small area, respectively, of erythema at the injection sites. In each subject, 1 μg. caused an erythematous area (30 to 35 mm. in diameter) to appear at the injection site, neighboring superficial veins became constricted, and a small central wheal appeared. The response reached a maximum in 10 to 12 minutes and persisted for over an hour. The injection of 10 μg. of serotonin intradermally provoked a similar response, except that erythema and venous constriction were evident over a wider area, and the effect lasted two hours. This response to intradermal serotonin was similar to that produced by intradermal antibody-lysed red cells, except that wheal formation was more prominent in the latter. The intravenous injection of 1 mg. of serotonin into a normal subject had no effect on leukocyte levels.

Taken together, the following observations strongly suggest that certain isoantibodies may, in causing hemolysis, provoke the release of histamine, serotonin and possibly other related substances from leukocytes and platelets: *1*) the finding of others (56–58) that antigen-antibody reactions with the probable involvement of complement cause a release of histamine and of serotonin from leukocytes, platelets and certain other cells; *2*) the observation of others (11) that red cell lysis *in vivo* by complement-fixing antibodies injures platelets as well as leukocytes; *3*) the observation made here that red cell hemolysates produced by complement-fixing antibodies provoke dermal reactions resembling those produced by histamine and serotonin; and finally, *4*) the observation made here that intravenous injections of small amounts of type A cells or of soluble A substance into type O recipients may cause an acute syndrome of facial flushing, dyspnea and hyperactive peristalsis. Similarly, during hemolysis by the complement-fixing autoantibodies of paroxysmal cold hemoglobinuria, urticaria often appears. Although attributed to a specific "dermolysin" (59) this urticaria is partly suppressed by antihistamines (60) and may reasonably be attributed to the sequence of events postulated to occur during hemolysis by isohemolysins.

That immune destruction of red cells, like other immune processes, may indirectly evoke certain tissue reactions is apparent in the ability of intravenously injected antibody-sensitized red cells to provoke the Shwartzman phenomenon in prepared skin sites of rabbits (61). It is of further interest that bacterial endotoxins, which prepare skin for the local Shwartzman reaction [which, in turn, involves the local formation of thrombi of platelets and leukocytes (*22*, *23*, 33)], induce a state of remarkable sensitivity to the action of serotonin (*62*). Davidsohn and his associates (63) have described suppurative skin lesions in rabbits at the site of injection of anti-rabbit-red cell hemolytic immune serum. The authors postulated that this effect reflected an antigenic similarity between red cells and skin; and, indeed, it is known that human epidermis may contain A and B antigens (64, 65). However, the skin reactions described here were minimal after direct injections of antiserum, and required for full development the presence of red cells. Thus, indirect, rather than direct, toxicity to skin was evidently involved.

A scheme summarizing the observed and postulated effects of immune hemolysis on the host is presented in Figure 14. Although certain striking distinctions have been emphasized in this report between the effects of hemolysis by anti-D and by anti-A or anti-B, it is believed that these distinctions are in part quantitative. Therefore, the two hemolytic systems are not differentiated in

[10] Kindly supplied by the Research Laboratories of the Upjohn Company, Kalamazoo, Mich.

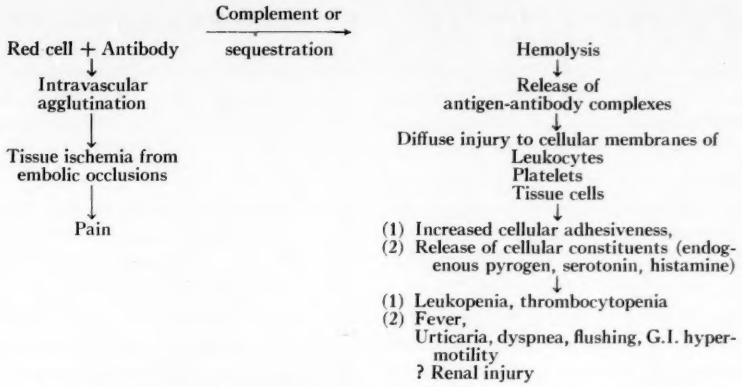

FIG. 14. PROPOSED MECHANISMS UNDERLYING SYSTEMIC REACTIONS TO HEMOLYSIS

Figure 14. However, it may be recalled in examining this scheme that the systemic effects of hemolysis by the ABO system primarily include certain immediate reactions, believed to reflect the effects of intravascular agglutination and of release of histamine and serotonin, whereas hemolysis by anti-D may be likened in its effects to substances such as tuberculin which cause delayed sensitivity, in which acute reactions are absent but in which fever is a striking feature. Finally, there is much evidence to support the contention that, just as anaphylaxis may arise from the release of histamine and serotonin from certain cells injured by the antigen-antibody reaction, fever following immune hemolysis may represent the action of endogenous pyrogen released from granulocytes (42, 44, 51, 52) similarly injured.

As noted earlier, it may be difficult, in considering acute hemolytic episodes, to ascertain to what extent anoxia, hemoglobinemia, embolization and shock may contribute to the injury sustained by the patient. Almost certainly, however, anaphylactic and pyrogenic phenomena contribute heavily to the acute damage,[11] and quite possibly to the more slowly apparent renal damage. It is doubtful that the renal shutdown observed in transfusion reactions or in paroxysms of cold hemoglobinuria (67) is caused by hemoglobinuria *per se,* unless this is preceded by functional tubular abnormalities (68). The danger of embolic occlusions in the kidney, as well as elsewhere, undoubtedly exists when hemolysis involves agglutinating antibodies, and focal cortical ischemia may be a prominent finding in the kidneys of patients dying of acute renal failure following mismatched transfusions (69). However, experimental efforts to demonstrate embolic intrarenal red cell agglutinates in animals during heterologous transfusion reactions have not been successful (70). It seems likely that the pyrogenic response, particularly when prolonged, may contribute to the pathogenesis of renal tubular injury by producing hypotension (71) and by direct derangement of renal function (72).

SUMMARY

Destruction of red cells by isoantibodies in normal human subjects was accompanied by a profound leukopenia, affecting granulocytes and monocytes. The abrupt, primarily hepatic, sequestration of ABO-incompatible red cells produced an abrupt leukopenia, while the slower, mainly splenic, sequestration of red cells coated with incomplete (anti-D) antibodies was accom-

[11] Accordingly, when symptoms of dyspnea, flushing and gastrointestinal disturbances occur very shortly after the onset of acute hemolysis, as by transfusion of incompatible blood, the use of parenteral agents which inhibit the action of histamine and serotonin (*e.g.,* chlorpromazine) may prove useful. In addition, antipyretics are certainly advisable in order to subdue the pyrogenic response, which generally occurs approximately an hour after the onset of hemolysis. It is interesting to note that, in the studies reported above, cortisone had no effect on the rate of blood destruction or on the leukopenia, but acted simply as an antipyretic (presumably on the central nervous system), as has been described in animals exposed to bacterial pyrogens (66).

panied by a synchronous, slowly developing leukopenia. In each instance there followed a leukocytosis, initially composed of immature granulocytes.

Leukopenia did not appear during hemolysis *in vitro* by nonimmunologic mechanisms involving either hepatic or splenic filtration of red cells. Although injections of soluble A substance into subjects whose sera contained anti-A did provoke leukopenia, the reaction of isoantibodies with red cells affected leukocyte levels only when actual red cell sequestration or lysis transpired.

Observations *in vitro* revealed that red cells coated with anti-D antibodies strongly adhered to leukocytes, particularly those of D-sensitized subjects. Similarly, mixed agglutinates containing leukocytes (regardless of blood type) and of red cells sensitized by ABO-incompatible plasma were encountered, confirming observations of others. In either instance, antibody-coated red cells adherent to leukocytes were transformed into spherocytes. Erythrophagocytosis was prominent only when red cells underwent direct hemolysis by complement. Despite the striking interactions between leukocytes and sensitized red cells observed *in vitro* and in the venous tourniquet-obstructed arms of normal subjects, there was no direct leukolysis; furthermore, the leukopenic effect probably did not result simply from the sequestration of mixed agglutinates.

The mechanism of the leukopenia (and possibly of the thrombocytopenia described by others) accompanying acute immune hemolysis, is believed to be analogous to that known to occur during certain other antigen-antibody reactions and following injections of bacterial endotoxins. Here, too, the injurious action on leukocytes (and platelets) of antigen-antibody complexes released from the hemolyzing red cell presumably causes the leukocytes to adhere to endothelial surfaces, particularly in organs with large capillary beds.

Febrile reactions, like leukopenia, accompanied hemolysis *in vivo* by immune, but not by nonimmune, mechanisms. Under the experimental conditions, marked fever occurred only during hemolysis by incomplete (anti-D) antibodies, and this occurred regardless of whether the interacting red cells or antibodies, or neither, were native to the subject. These reactions were characteristic of pyrogenic responses in general in their delayed appearance and were also analogous to reactions to bacterial pyrogens in that slow removal of the "pyrogen" from the blood stream provoked the more severe febrile responses.

The intracutaneous injection of red cells into ABO-incompatible subjects manifesting hemolysins for these red cells *in vitro* provoked immediate, transient, local skin reactions consisting of wheal formation, erythema and venous constriction. Such reactions also followed intracutaneous injections of autogenous red cells hemolyzed *in vitro* beforehand by isoantibodies. It is believed that these dermal effects represent the release of histamine, serotonin and related compounds from leukocytes and platelets injured by immune hemolysis.

On the basis of these findings it is suggested that many of the systemic effects of immune hemolysis (leukopenia, thrombocytopenia, chills and fever, dyspnea, flushing, urticaria, gastrointestinal disturbances, and possibly renal injury) reflect in part the injurious effects of the released antigen-antibody complexes on leukocytes and platelets. This injury is believed to consist of a change in the surface properties of these cells and a release of certain of their constituents including endogenous pyrogen, histamine, and serotonin.

REFERENCES

1. Castle, W. B., Ham, T. H., and Shen, S. C. Observations on the mechanism of hemolytic transfusion reactions occurring without demonstrable hemolysin. Trans. Ass. Amer. Phycns 1950, **63**, 161.
2. Jandl, J. H., Jones, A. R., and Castle, W. B. The destruction of red cells by antibodies in man. I. Observations on the sequestration and lysis of red cells altered by immune mechanisms. J. clin. Invest. 1957, **36**, 1428.
3. Jandl, J. H., and Castle, W. B. Agglutination of sensitized red cells by large anisometric molecules. J. Lab. clin. Med. 1956, **47**, 669.
4. Jandl, J. H. Sequestration of reticulocytes and of abnormal red cells by filtration at low pressures (abstract). J. clin. Invest. 1958, **37**, 905.
5. Wintrobe, M. M. Clinical Hematology, 4th ed. Philadelphia, Lea and Febiger, 1956.
6. Brittingham, T. E., and Chaplin, H., Jr. Sensitivity to buffy coat—A cause of "non-specific" transfusion reactions (abstract). J. clin. Invest. 1957, **36**, 877.
7. Widal, F., Abrami, P., and Brissaud, E. L'auto-anaphylaxie; Son rôle dans l'hémoglobinurie paroxystique; Traitement anti-anaphylactique de

l'hémoglobinurie; Conception physique de l'anaphylaxie. Sem. méd. (Paris) 1913, **33**, 613.

8. Bjørn-Hansen, H. Über die paroxysmale Kältehämoglobinurie. Acta med. scand. 1936, **88**, 129.
9. Young, L. E., Ervin, D. M., and Yuile, C. L. Hemolytic reactions produced in dogs by transfusion of incompatible dog blood and plasma. I. Serologic and hematologic aspects. Blood 1949, **4**, 1218.
10. Bierman, H. R., Kelly, K. H., Byron, R. L., Jr., Cordes, F. L., White, L. P., and Littman, A. Auto-removal of leukocytes from the peripheral circulation of man initiated by administration of anti-group specific substances (abstract). Amer. J. Med. 1953, **15**, 410.
11. Swisher, S. N. Nonspecific adherence of platelets and leukocytes to antibody-sensitized red cells: A mechanism producing thrombocytopenia and leukopenia during incompatible transfusions (abstract). J. clin. Invest. 1956, **35**, 738.
12. Bakemeier, R. F., and Swisher, S. N. Mixed agglutination of leukocytes and erythrocytes in relation to studies of leukocyte antigens. Blood 1957, **12**, 913.
13. Jandl, J. H. Pyrogenic and leukopenic effects of immune hemolysis in man (abstract). J. clin. Invest. 1957, **36**, 904.
14. Jandl, J. H., Greenberg, M. S., Yonemoto, R. H., and Castle, W. B. Clinical determination of the sites of red cell sequestration in hemolytic anemias. J. clin. Invest. 1956, **35**, 842.
15. Ham, T. H. A Syllabus of Laboratory Examinations in Clinical Diagnosis. Cambridge, Harvard University Press, 1950.
16. Coombs, R. R. A., Mourant, A. E., and Race, R. R. A new test for the detection of weak and "incomplete" Rh agglutinins. Brit. J. exp. Path. 1945, **26**, 255.
17. Shen, S. C., and Ham, T. H. Studies on the destruction of red blood cells. III. Mechanism and complications of hemoglobinuria in patients with thermal burns; spherocytosis and increased osmotic fragility of red blood cells. New Engl. J. Med. 1943, **229**, 701.
18. Zieve, L., Hill, E., Hanson, M., Falcone, A. B., and Watson, C. J. Normal and abnormal variations and clinical significance of the one-minute and total serum bilirubin determinations. J. Lab. clin. Med. 1951, **38**, 446.
19. Quick, A. J. The Physiology and Pathology of Hemostasis. Philadelphia, Lea and Febiger, 1951.
20. Biedl, A., and Kraus, R. Experimentelle Studien über Anaphylaxie. Wien. klin. Wschr. 1909, **22**, 363.
21. Dean, H. R., and Webb, R. A. The blood changes in anaphylactic shock in the dog. J. Path. Bact. 1924, **27**, 65.
22. Stetson, C. A., Jr. Studies on the mechanism of the Shwartzman phenomenon; certain factors involved in the production of local hemorrhagic necrosis. J. exp. Med. 1951, **93**, 489.
23. Stetson, C. A., Jr. Studies on the mechanism of the Shwartzman phenomenon; similarities between reactions to endotoxins and certain reactions of bacterial allergy. J. exp. Med. 1955, **101**, 421.
24. Kopeloff, N., and Kopeloff, L. M. Blood platelets in anaphylaxis. J. Immunol. 1940, **40**, 471.
25. Roemer, F. Darstellung und Wirkung proteinhältiger Bakterienextrakte. Berl. klin. Wschr. 1891, **28**, 1189.
26. Goldscheider, A., and Jacob, P. Ueber die Variationen der Leukocytose. Z. klin. Med. 1894, **25**, 373.
27. Andrewes, F. W. The behaviour of the leucocytes in infection and immunity. Lancet 1910, **2**, 8.
28. Scully, F. J. The reaction after intravenous injections of foreign protein. J. Amer. med. Ass. 1917, **69**, 20.
29. Bennett, I. L., Jr., and Beeson, P. B. The properties and biologic effects of bacterial pyrogens. Medicine 1950, **29**, 365.
30. Thomas, L. The physiological disturbances produced by endotoxins. Ann. Rev. Physiol. 1954, **16**, 467.
31. Butler, J. J. Some studies on the naturally occurring leucocyte agglutinins. J. clin. Invest. 1956, **35**, 1150.
32. Abell, R. G., and Schenck, H. P. Microscopic observations on the behavior of living blood vessels of the rabbit during the reaction of anaphylaxis. J. Immunol. 1938, **34**, 195.
33. Stetson, C. A., Jr. Similarities in the mechanisms determining the Arthus and Shwartzman phenomena. J. exp. Med. 1951, **94**, 347.
34. Berthrong, M., and Cluff, L. E. Studies of the effect of bacterial endotoxins on rabbit leucocytes; I. Effect of intravenous injection of the substances with and without induction of the local Shwartzman reaction. J. exp. Med. 1953, **98**, 331.
35. Webb, R. A. The mechanism of anaphylactic leucopenia in dogs. J. Path. Bact. 1924, **27**, 79.
36. Tocantins, L. M. The mammalian blood platelet in health and disease. Medicine 1938, **17**, 155.
37. Craddock, C. G., Jr., Perry, S., and Lawrence, J. S. The dynamics of leukopoiesis and leukocytosis, as studied by leukopheresis and isotopic techniques. J. clin. Invest. 1956, **35**, 285.
38. Perry, S., Weinstein, I. M., Craddock, C. G., and Lawrence, J. S. The origin of cells contributing to leukocytosis. Alterations in white cell physiology induced by typhoid as compared to leukophoresis *in* Proc. Sixth Internat. Cong. Hematology. New York, Grune and Stratton, 1958.
39. Zinkham, W. H., and Diamond, L. K. In vitro erythrophagocytosis in acquired hemolytic anemia. Blood 1952, **7**, 592.
40. Turner, J. C. Absence of lecithin from the stromata of the red cells of certain animals (ruminants),

and its relation to venom hemolysis. J. exp. Med. 1957, **105**, 189.

41. Dameshek, W., and Schwartz, S. O. Acute hemolytic anemia (acquired hemolytic icterus, acute type). Medicine 1940, **19**, 231.
42. Bennett, I. L., Jr., and Beeson, P. B. Studies on the pathogenesis of fever. I. The effect of injection of extracts and suspensions of uninfected rabbit tissues upon the body temperature of normal rabbits. J. exp. Med. 1953, **98**, 477.
43. Beeson, P. B. Tolerance to bacterial pyrogens. II. Role of the reticulo-endothelial system. J. exp. Med. 1947, **86**, 39.
44. Atkins, E., and Wood, W. B., Jr. Studies on the pathogenesis of fever. II. Identification of an endogenous pyrogen in the blood stream following the injection of typhoid vaccine. J. exp. Med. 1955, **102**, 499.
45. Braude, A. I., Carey, F. J., and Zalesky, M. Investigation of tolerance to bacterial endotoxin with radiochromium labelled *E. coli* endotoxin (abstract). J. clin. Invest. 1955, **34**, 923.
46. Rowley, D. A. The formation of circulating antibody in the splenectomized human being following intravenous injection of heterologous erythrocytes. J. Immunol. 1950, **65**, 515.
47. Rowley, D. A. The effect of splenectomy on the formation of circulating antibody in the adult male albino rat. J. Immunol. 1950, **64**, 289.
48. Levine, P. The influence of the ABO system on Rh hemolytic disease. Hum. Biol. 1958, **30**, 14.
49. Stern, K., Davidsohn, I., and Masaitis, L. Experimental studies on Rh immunization. Amer. J. clin. Path. 1956, **26**, 833.
50. Bennett, I. L., Jr., Petersdorf, R. G., and Keene, W. R. Pathogenesis of fever: Evidence for direct cerebral action of bacterial endotoxins. Trans. Ass. Amer. Phycns 1957, **70**, 64.
51. Cranston, W. I., Goodale, F., Jr., Snell, E. S., and Wendt, F. The role of leucocytes in the initial action of bacterial pyrogens in man. Clin. Sci. 1956, **15**, 219.
52. Snell, E. S., Goodale, F., Jr., Wendt, F., and Cranston, W. I. Properties of human endogenous pyrogen. Clin. Sci. 1957, **16**, 615.
53. Farr, R. S. The febrile response upon injection of bovine albumin into previously sensitized rabbits (abstract). J. clin. Invest. 1958, **37**, 894.
54. Magalini, S. I., and Stefanini, M. Platelets XVIII. Relationship of platelets to activity of 5-hydroxytryptamine creatinine sulfate. Proc. Soc. exp. Biol. (N. Y.) 1956, **92**, 788.
55. Rowley, D. A., and Benditt, E. P. 5-Hydroxytryptamine and histamine as mediators of the vascular injury produced by agents which damage mast cells in rats. J. exp. Med. 1956, **103**, 399.
56. Humphrey, J. H., and Jaques, R. The release of histamine and 5-hydroxytryptamine (serotonin) from platelets by antigen-antibody reactions (*in vitro*). J. Physiol. 1955, **128**, 9.
57. Katz, G. Histamine release from blood cells in anaphylaxis in vitro. Science 1940, **91**, 221.
58. Waalkes, T. P., Weissbach, H., Bozicevich, J., and Udenfriend, S. Serotonin and histamine release during anaphylaxis in the rabbit. J. clin. Invest. 1957, **36**, 1115.
59. Harris, K. E., Lewis, T., and Vaughan, J. M. Haemoglobinuria and urticaria from cold occurring singly or in combination; observations referring especially to the mechanism of urticaria with some remarks upon Raynaud's disease. Heart 1929, **14**, 305.
60. Becker, R. M. Paroxysmal cold hemoglobinurias. Arch. intern. Med. 1948, **81**, 630.
61. Shwartzman, G. Phenomenon of Local Tissue Reactivity and Its Immunological, Pathological and Clinical Significance. New York, Paul B. Hoeber, Inc., 1937.
62. Thomas, L., Zweifach, B. W., and Benacerraf, B. Mechanisms in the production of tissue damage and shock by endotoxins. Trans. Ass. Amer. Phycns 1957, **70**, 54.
63. Davidsohn, I., Hanawalt, E., Goldman, S., Hermoni, D., and Muratore, R. Skin toxicity of a hemolytic serum. Arch. Path. 1957, **64**, 540.
64. Coombs, R. R. A., Bedford, D., and Rouillard, L. M. A and B blood-group antigens on human epidermal cells demonstrated by mixed agglutination. Lancet 1956, **1**, 461.
65. Nelken, D., Gurevitch, J., and Neuman, Z. A and B antigens in the human epidermis. J. clin. Invest. 1957, **36**, 749.
66. Kass, E. H., and Finland, M. Effect of ACTH on induced fever. New Engl. J. Med. 1950, **243**, 693.
67. Stevenson, I. P. Paroxysmal hemoglobinuria with report of a case. McGill med. J. 1943, **12**, 192.
68. Lalich, J. J., and Schwartz, S. I. The role of aciduria in the development of hemoglobinuric nephrosis in dehydrated rabbits. J. exp. Med. 1950, **92**, 11.
69. Oliver, J., MacDowell, M., and Tracey, A. The pathogenesis of acute renal failure associated with traumatic and toxic injury. Renal ischemia, nephrotoxic damage and the ischemuric episode. J. clin. Invest. 1951, **30**, 1307.
70. Govaerts, P., Desclin, L., and Desclin, R. Expériences concernant le rôle éventuel des agglutinats de globules rouges dans les accidents rénaux consécutifs à la transfusion de sang incompatible. Rev. belge Path. 1956, **25**, 286.
71. Bradley, S. E., Chasis, H., Goldring, W., and Smith, H. W. Hemodynamic alterations in normotensive and hypertensive subjects during the pyrogenic reaction. J. clin. Invest. 1945, **24**, 749.
72. Lathem, W. The urinary excretion of sodium and potassium during the pyrogenic reaction in man. J. clin. Invest. 1956, **35**, 947.